

**THE CONTRIBUTION OF MOLECULAR CHAPERONES TO THE ER-ASSOCIATED  
DEGRADATION OF APOLIPOPROTEIN B IN BOTH YEAST AND MAMMALIAN  
SYSTEMS**

by

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# **THE CONTRIBUTION OF MOLECULAR CHAPERONES TO THE ER-ASSOCIATED DEGRADATION OF APOLIPOPROTEIN B IN BOTH YEAST AND MAMMALIAN SYSTEMS**

Stacy Lynn Hrizo, PhD  
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Apolipoprotein B (ApoB) is the major structural protein component of chylomicrons and very low and low density lipoproteins, the major cholesterol carrying particles in the blood. High levels of ApoB have been directly linked to the risk of developing coronary artery disease. Pre-secretory degradation is one important mechanism regulating the assembly and secretion of ApoB.

A major pre-secretory degradation pathway regulating ApoB production is Endoplasmic Reticulum Associated Degradation (ERAD), a pathway in which molecular chaperones play key roles. Molecular chaperones assist with protein folding, assembly, translocation, and targeting misfolded proteins to the proteasome for degradation. Previous work in mammalian cells and using cell-free systems with yeast cytosols established that the cytosolic chaperones, Hsp70 and Hsp90, promote ApoB ERAD.

To identify additional chaperones involved in ApoB ERAD, I employed a cell-free system in which Hsp70, Hsp90 and proteasome-dependent degradation of the ApoB48 isoform is recapitulated with yeast cytosol. In addition, I developed an *in vivo* yeast expression system for the ApoB29 isoform. The shorter ApoB29 isoform is localized to the ER and associates with chaperones in yeast. Using both the *in vitro* and *in vivo* systems I found that a yeast Hsp110, Sse1p, associates with and stabilizes ApoB, which contrasts with data indicating that Hsp70 and Hsp90 facilitate ApoB degradation. Because Sse1p is reported to associate with Ssa1p, an

Hsp70 known to regulate ApoB degradation in vitro, and Ssb1p and Ssb2p, Hsp70s in the ribosome associated complex (RAC), I tested the contributions of Ssb1p and Ssb2p on ApoB ERAD in vitro and in vivo but observed no difference in ApoB degradation rates compared to wildtype strains. Together, these data indicate that Sse1p, but not RAC, contributes to ApoB stabilization. To determine whether my results are relevant in mammalian cells, Hsp110 was over-expressed in hepatocytes and enhanced ApoB secretion was observed. This study indicates that chaperones within distinct complexes can play unique roles during ER-associated degradation (ERAD), establishes a role for Sse1/Hsp110 in ERAD, and identifies Hsp110 as a target to lower cholesterol.

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## **PREFACE**

Scientific research is not a solitary activity and the work described in this document could not have been completed without the assistance of many talented colleagues and friends throughout my graduate career. First, I would like to thank my advisor, Dr. Jeffrey L. Brodsky, who is a wonderful mentor who always had helpful suggestions and ideas as my research project developed. More importantly, he has cultivated a wonderful research environment in his laboratory that fosters independent thinking with an ample support system for those days when trouble-shooting is necessary. A chief component of that laboratory environment is the lab manager, Jen Goeckeler, who is not only has made my scientific life extremely easy by ensuring that research materials are always available, but who has also become a great friend with whom I can chat about data, life and music.

All of the past and present members of the Brodsky lab are wonderful individuals and I feel honored to have worked with all of them. I wanted to specifically mention Dr. Christine Wright, a previous graduate student, and Shruthi Vembar, Sandlin Seguin, and Karen Hecht, current graduate students, and Dr. Teresa Buck, a current post-doctoral fellow, whose friendship has been invaluable. A previous post-doctoral fellow in the lab, Dr. Sheara Fewell, was instrumental in helping me successfully clone ApoB into a yeast expression vector. I also want to thank my research bay mate, Dr. Kunio Nakatsukasa, for his scientific advice, I do not know

anyone with better research hands. In addition, I want to thank Donald Schleicher, Jacob McBride, Sara Herrle, and Nick Simon for their work on the ApoB project. It was a truly wonderful experience teaching them about the ApoB in vitro and in vivo systems and some of the data they produced is included in this document. I also want to acknowledge my teaching mentors Drs. Melanie Popa and Nancy Kaufmann. They are knowledgeable instructors that have always given me time and advice as I have been developing and implementing my own teaching philosophy in the classroom.

I have had the great experience of collaborating with Dr. Edward Fisher's laboratory at New York University School of Medicine. Dr. Fisher and his research staff have been invaluable sources of information and materials during the development of this research project. In addition, Dr. Fisher has generously opened the door of his lab to me twice so that I could conduct experiments using systems developed by his research group. Specifically, Dr. Viktoria Gusarova was the post-doctoral fellow who originally developed the in vitro ApoB degradation assay and graciously took time out of her schedule to teach the system to me. Furthermore, the mammalian Hsp110 overexpression experiments would not have been possible without the work of David Habel and Dr. Viktoria Gusarova and the helpful instruction of Dr. Ursula Andreo.

I want to acknowledge the support and help that my family and husband have provided for me during my entire educational experience. My mother and father always instilled in me a belief that any goal is attainable. My family has been a loving support system, attending every major educational milestone. I want to specifically acknowledge my brother, Mark, who is a Pitt alumni and who is extremely happy I went to Pitt for my graduate work. Finally, I want to acknowledge my husband, John, who makes every day an adventure and is my loudest cheerleader. Thank you all for your help and support over the years!

## **1.0 INTRODUCTION**

### **1.1 APOLIPOPROTEIN B**

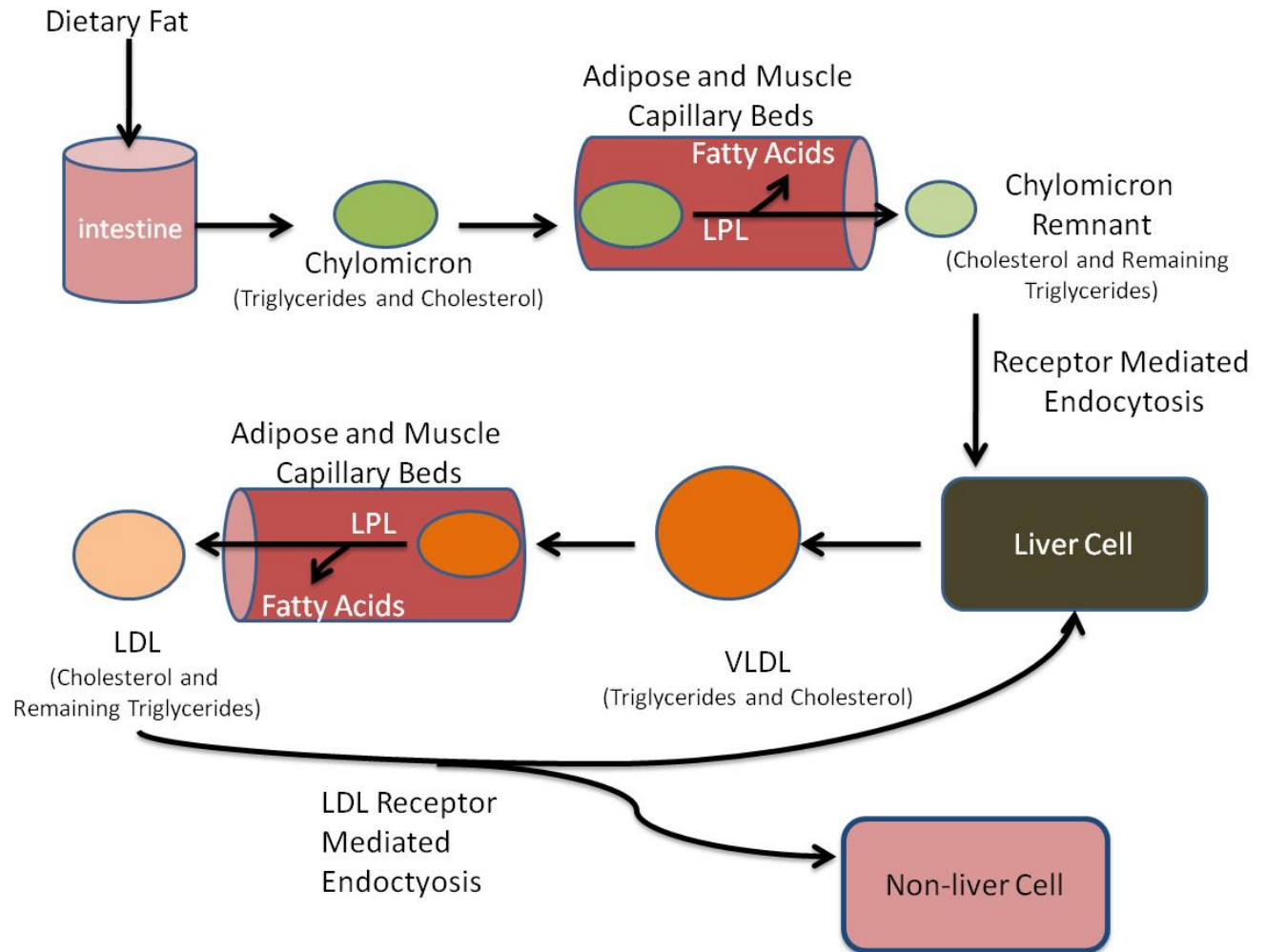
Apolipoprotein B (ApoB) is the major structural protein in chylomicrons, very low density (VLDL) and low density (LDL) lipoprotein particles, the cholesterol and lipid carrying particles in the blood. Chylomicrons are secreted by the small intestine, carry dietary fatty nutrients and contain the ApoB48 isoform, which is 48% the size of the full length ApoB protein. VLDL and LDL particles are synthesized and secreted from the liver and carry excess dietary and endogenously synthesized cholesterol and lipids. ApoB100, the full length protein, is the isoform found in VLDL and LDL particles. Cumulatively, chylomicrons, VLDL and LDL particles are essential for delivery of dietary and endogenously synthesized cholesterol and lipids to peripheral tissues. The fatty nutrients transported by ApoB are required for a variety of cellular functions such as cell membrane synthesis, the regulation of cell membrane permeability via cholesterol content and lipid composition, bile acid synthesis, and cellular metabolism (For recent reviews on ApoB see (Brodsky et al., 2004; Davis, 1999; Fisher and Ginsberg, 2002; Olofsson and Boren, 2005; Segrest et al., 2001)).

### 1.1.1 Lipid Cycle And Disease

The lipoprotein particles are intricate structures consisting of a core of neutral lipids (triglycerides and/or cholesterol esters), which are surrounded by a monolayer of amphipathic lipids (phospholipids and unesterified cholesterol). The amphipathic nature of the amino acid sequence of ApoB allows the polypeptide to interact with both hydrophobic and hydrophilic environments; this characteristic allows the protein to intimately associate with the fatty nutrients and thus function as a detergent for these hydrophobic molecules in the bloodstream (Segrest et al., 1994). The overall process by which these lipoprotein particles are synthesized, secreted, circulated, and endocytosed in the organism is generally referred to as the lipid cycle and will be described in the following paragraphs (Figure 1).

Dietary cholesterol and triglycerides are packaged into chylomicron particles with ApoB48 in the small intestine. These chylomicrons are subsequently secreted from enterocytes into the lymphatic system where they then travel to the bloodstream. In the capillary beds of muscle and adipose tissues the triglyceride core of the chylomicron is hydrolyzed into fatty acids and glycerol by lipoprotein lipase (LPL) and the co-factor apolipoprotein C-II (ApoC-II) (Havel, 1975; Kwiterovich, 2000; Mjos et al., 1975). These fatty acids are converted back into triglycerides for storage in adipose cells and are utilized in metabolic pathways for energy in muscle cells. The triglyceride depleted chylomicron, which is referred to as a remnant, will be endocytosed into a liver cell (Kita et al., 1982; Rohlmann et al., 1998; Rubinsztein et al., 1990). The dietary cholesterol and remaining triglycerides are repackaged into a VLDL particle with endogenously synthesized fatty nutrients to be released into the bloodstream (Hamilton, 1972; Kwiterovich, 2000).





### **Figure 1: The Lipid Cycle**

This diagram depicts the circulation of both exogenous (dietary) and endogenous fatty nutrients by lipoprotein particles. Dietary lipids and cholesterol are absorbed through the wall of the small intestine and packaged into chylomicrons. These chylomicrons deliver fatty acids released by lipoprotein lipase (LPL) to skeletal muscle and adipose tissue. The cholesterol rich chylomicron remnant is endocytosed into the liver. The dietary cholesterol is then repackaged into a VLDL particle with endogenously synthesized fatty nutrient and secreted. Again the triglycerides are removed by LPL in the capillary beds of adipose tissue and skeletal muscle. This density of the VLDL particle increases transitioning the particle to an LDL particle. The LDL particle can either be taken up by receptor mediated endocytosis into peripheral tissues for use in membrane synthesis and metabolism or liver cells for repackaging into a VLDL particle. (Adapted from (Brown and Goldstein, 1984))

Similar to chylomicrons the newly secreted VLDL particle is depleted of its triglyceride content by LPL and ApoC-II in the capillary beds of muscle and adipose tissues (Havel, 1975; Kwiterovich, 2000; Mjos et al., 1975). The decrease in triglyceride content increases the density of the particle that is now referred to as an LDL particle. The reduction in the lipid content of the VLDL particle by LPL exposes a portion of the ApoB polypeptide that was previously buried by lipids. The newly exposed portion of ApoB functions as a binding site for the LDL receptor that is required for receptor mediated endocytosis of ApoB into the target cell (Boren et al., 1998). The LDL receptor is expressed on hepatocytes and selectively expressed on peripheral tissues that require cholesterol for new membrane synthesis, thus the LDL particle is only endocytosed by cells that are competent for cholesterol and fatty acid metabolism (Brown and Goldstein, 1975; Brown and Goldstein, 1976a; Brown and Goldstein, 1976b; Herz et al., 1988).

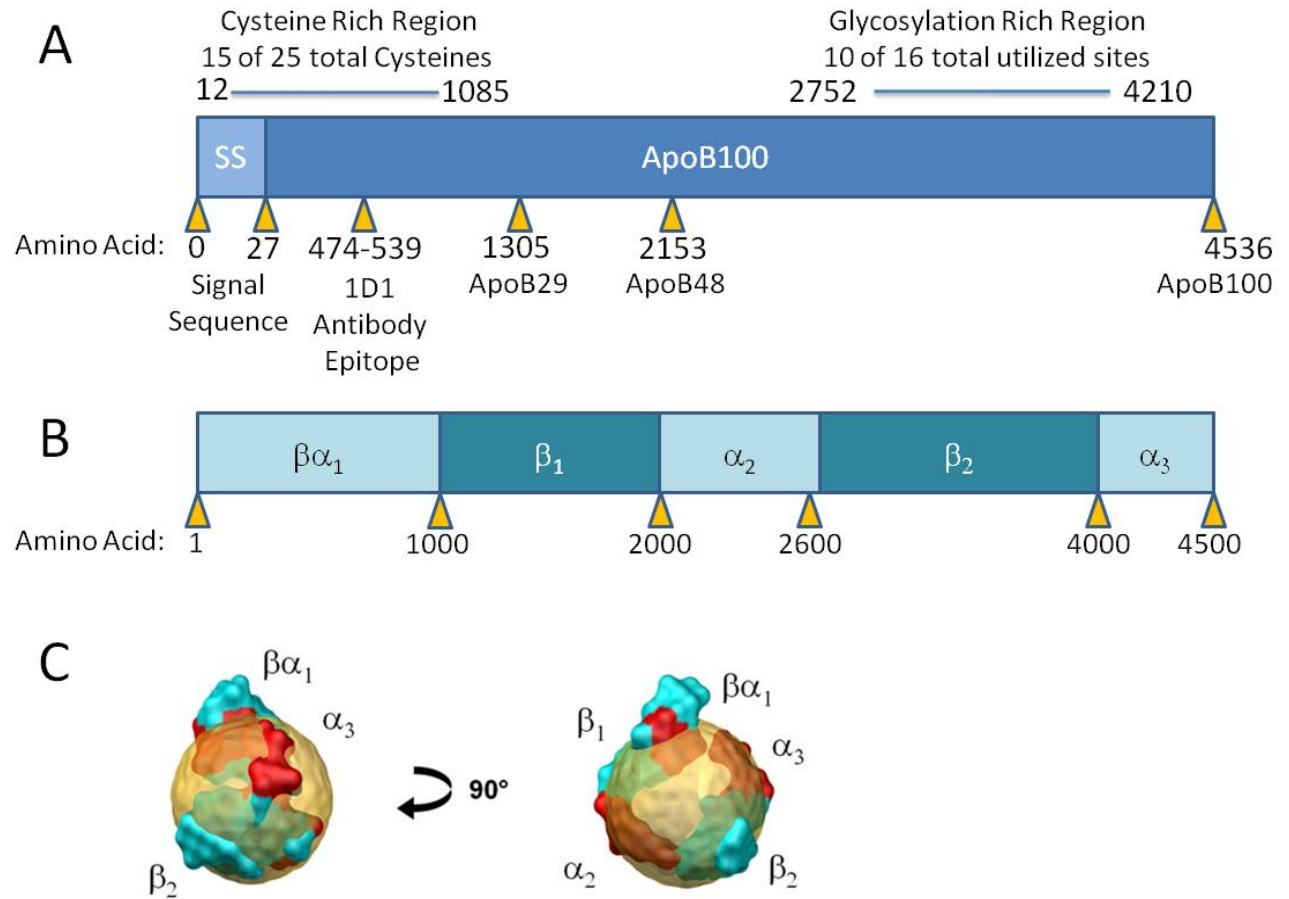
A loss of regulation of synthesis, secretion, or uptake of these ApoB lipoprotein particles from the bloodstream is known to result in various disease states. Low circulating VLDL levels is indicative of a disease state known as hypobetalipoproteinemia, in which patients' exhibit characteristics associated with fat malabsorption such as ataxia, neuromuscular degeneration, retinitis pigmentosa, and fat soluble vitamin deficiency. These phenotypes are due to the starvation of peripheral tissues for cholesterol and fatty acids for membrane biogenesis. In addition, the patients develop a fatty liver as a result of the inability of the hepatocytes to secrete the endogenously synthesized fatty nutrients in lipoprotein particles, thus overloading the tissue with lipids and cholesterol (Linton et al., 1993; Whitfield et al., 2004).

Not only is a reduction in the circulating ApoB-containing lipoprotein particle levels linked to human disease, but more commonly high circulating levels of ApoB correlates with a high risk for the development of atherosclerotic plaques and subsequently coronary artery

disease, a leading cause of death in North America (Kannel et al., 1971). Many cases of familial coronary artery disease have been linked to a mutation in the LDL receptor which reduces the ability of the receptor to interact with the LDL particle (Goldstein et al., 1982). As a result of reduced receptor-ApoB interactions, the amount of LDL particles circulating in the blood stream of the patient increases. Other factors associated with developing high LDL serum content are a high fat diet, mutations of LPL that result in reduced function of the triglyceride metabolizing enzyme, and elevated expression of apolipoprotein C-3, an inhibitor of LPL (Kwiterovich, 2000). High LDL content in the blood enhances the risk that the particle may be absorbed into the endothelial lining of the blood vessel wall and may become oxidized (Camejo et al., 1998; Kume et al., 1992; Leitinger, 2003; Skalen et al., 2002; Watson et al., 1997). The oxidized LDL particle can be taken up by macrophages which then become transformed into foam cells (Smith et al., 1995b). The foam cells and surrounding endothelial cells secrete growth factors that stimulate the proliferation and migration of arterial smooth muscle cells. The inflammatory response generated by the presence of oxidized LDL particles in the arterial wall ultimately generates an atherosclerotic lesion (Leitinger, 2003; Olofsson and Boren, 2005). These lesions reduce the diameter of the blood vessel and increase the patient's risk for a heart attack or stroke. Overall, understanding the regulation of synthesis, secretion and uptake of lipoprotein particles is a key area of research for the treatment and prevention of both hypobetalipoproteinemia and coronary artery disease.

### **1.1.2 Structural Features Of Apolipoprotein B**

ApoB100, the isoform produced by hepatic cells, is a long polypeptide (>4500 amino acids) and is present as a single molecule per lipoprotein particle (Figure 2) (Chen et al., 1986). The



## **Figure 2: Apolipoprotein B Isoforms And Model Of ApoB Structure In a LDL Particle**

A: This linear diagram of ApoB depicts the length of the 3 isoforms of ApoB utilized in my studies: ApoB29, ApoB48, and ApoB100. The ER targeting sequence (signal sequence) consists of amino acids 1-27. The ID1 binding site (amino acids 474-539) is the location of the epitope that the ApoB antibody used in my experiments recognized for detection by immunoblot. ApoB has 25 total cysteine residues (amino acid residue: 12, 51, 61, 70, 159, 185, 218, 234, 358, 363, 451, 486, 939, 949, 1085, 1395, 1478, 1635, 2906, 3167, 3297, 3734, 3890, 4190, 4326) in the full length polypeptide and 16 of them are involved in disulfide bonds in the folded protein structure (Yang et al., 1990). There is a cysteine rich region (12-1085aa) in the polypeptide that contains 15 of the cysteine residues and seven of the disulfide bonds. ApoB also contains 16 confirmed N-linked glycosylation sites (amino acid residue: 158, 956, 1341, 1350, 1496, 2752, 2955, 3074, 3197, 3309, 3331, 3384, 3438, 3438, 3868, 4210, 4404) and ten of them are localized to the last 1500 amino acids of the full length ApoB100 polypeptide (Harazono et al., 2005).

B: Linear diagram of the proposed secondary structures of ApoB in a VLDL particle. The amino acid residues that comprise each domain are as follows (Johs et al., 2006):  $\beta\alpha 1$  domain (residues 1-1000);  $\beta 1$  domain (residues 1000-2000);  $\alpha 2$  domain (2100-2600);  $\beta 2$  domain (residues 2600-4000);  $\alpha 3$  domain (4100-4500). The proline rich domain (prd1) consists of amino acid residues 3345-3381 and functions as the LDL receptor interaction domain (Segrest et al., 2001).

C: Model of ApoB secondary structure in an LDL particle from Johs, et al., 2006. Notice the highly disulfide bonded  $\beta\alpha 1$  domain is a globular structure that is not as tightly associated with lipid as the other domains of ApoB (Johs et al., 2006).

ApoB48 isoform, the N-terminal 48% of ApoB100, (Figure 2), is expressed in enterocytes. ApoB48 is produced by post-transcriptional modification of the ApoB mRNA at codon 2153 that converts a glutamine codon (CAA) to a stop codon (UAA) (Chen et al., 1987). The enzyme responsible for this conversion is an RNA-specific cytidine deaminase, ApoB mRNA editing enzyme catalytic complex (Apobec-1). Apobec-1 is only expressed in intestinal epithelial cells: therefore, it is the post-transcriptional editing process that determines if an ApoB48 containing chylomicron or ApoB100 containing VLDL particle is produced (Navaratnam et al., 1993).

Another isoform that is often utilized and was particularly important to my research is ApoB29 (29% the size of full length ApoB, 1305 amino acids) (Figure 2). This isoform was originally identified in hypobetalipoproteinemia patients. The ApoB gene contained a C to T transition that encoded a premature stop in their ApoB gene converting the transcript from ApoB100 to ApoB29 (Collins et al., 1988; Huang et al., 1989). Subsequent research has found that the truncated ApoB29 isoform forms smaller, denser, lipid poor particles. The shorter length of the mutant ApoB29 isoform limits the amount of fatty nutrients that can associate with the polypeptide to form a lipoprotein particle (Linton et al., 1993). Additional studies that focused on the trafficking and secretion of ApoB truncation mutants reported that ApoB29 is the smallest isoform of ApoB that can traffic normally through hepatic cells to form a lipoprotein particle. Smaller truncations have been found to have reduced secretion rates from hepatic cell lines (McLeod et al., 1996). Therefore, ApoB29 is the smallest clinically relevant isoform of ApoB that can be examined for normal trafficking and turnover in hepatic cells.

The amphipathic nature of ApoB is essential for its function. The hydrophobic regions are necessary to noncovalently interact with the hydrophobic fatty nutrients to facilitate their transport while retaining the solubility of the particle in the hydrophilic environment of the

blood. Other regions are important for targeting lipoprotein particles to peripheral tissues and to the liver for uptake by receptor mediated endocytosis. Residues 3345-3381 in the protein have been identified as the LDL receptor binding domain. This was established by sequence comparison in seven different species, antibody binding experiments (this site is occluded from antibody binding while interacting with the receptor) and by truncation analysis (ApoB75 and shorter isoforms did not interact with the LDL receptor) (Boren et al., 1998; Law and Scott, 1990; Milne et al., 1989; Segrest et al., 2001). As described previously, LDL receptor site binding is vital for clearance from the blood by the peripheral tissues after the site is exposed by metabolic conversion from VLDL to an LDL particle.

The current model for the secondary structure of ApoB in the lipoprotein molecule is a series of amphipathic alpha-helices and beta-sheet domains designated:  $\text{NH}_2\text{-}\beta\alpha_1\text{-}\beta_1\text{-}\alpha_2\text{-}\beta_2\text{-}\alpha_3\text{-COOH}$  (Segrest et al., 1994). The modeled secondary structure of ApoB based on data from the crystal structure of the lipid associated nine stranded amphipathic  $\beta$ -sheet domains of Lamprey lipovitellin (this protein has 23% similarity to the  $\beta$  sheet domains of ApoB) and on small angle neutron scattering data from detergent solubilized ApoB. The structural data predicts that the  $\beta_1$  and  $\beta_2$  domains are intimately associated with the lipid core, while the  $\beta\alpha_1$  domain does not appear to tightly contact the fatty nutrient core and forms a more globular structure associated with the particle (Johs et al., 2006; Segrest et al., 2001) (Figure 2).

A unique feature of the N-terminal  $\beta\alpha_1$  domain is that it contains 14 of the 25 total cysteines in ApoB100 and all of them are involved in disulfide bond formation (Figure 1). This accounts for seven of the eight disulfide bonds found in ApoB100 (Burch and Herscovitz, 2000; Yang et al., 1990). The high disulfide bond content is predicted to make this region of ApoB highly compact and globular and matches well with the current structural data (Figure 2) (Johs et



al., 2006; Segrest et al., 2001; Segrest et al., 1994). The presence of a disulfide bond rich N-terminus is highly important for the secretion of ApoB and when the formation of the disulfide bonds is blocked a reduction in ApoB secretion is observed (Burch and Herscovitz, 2000).

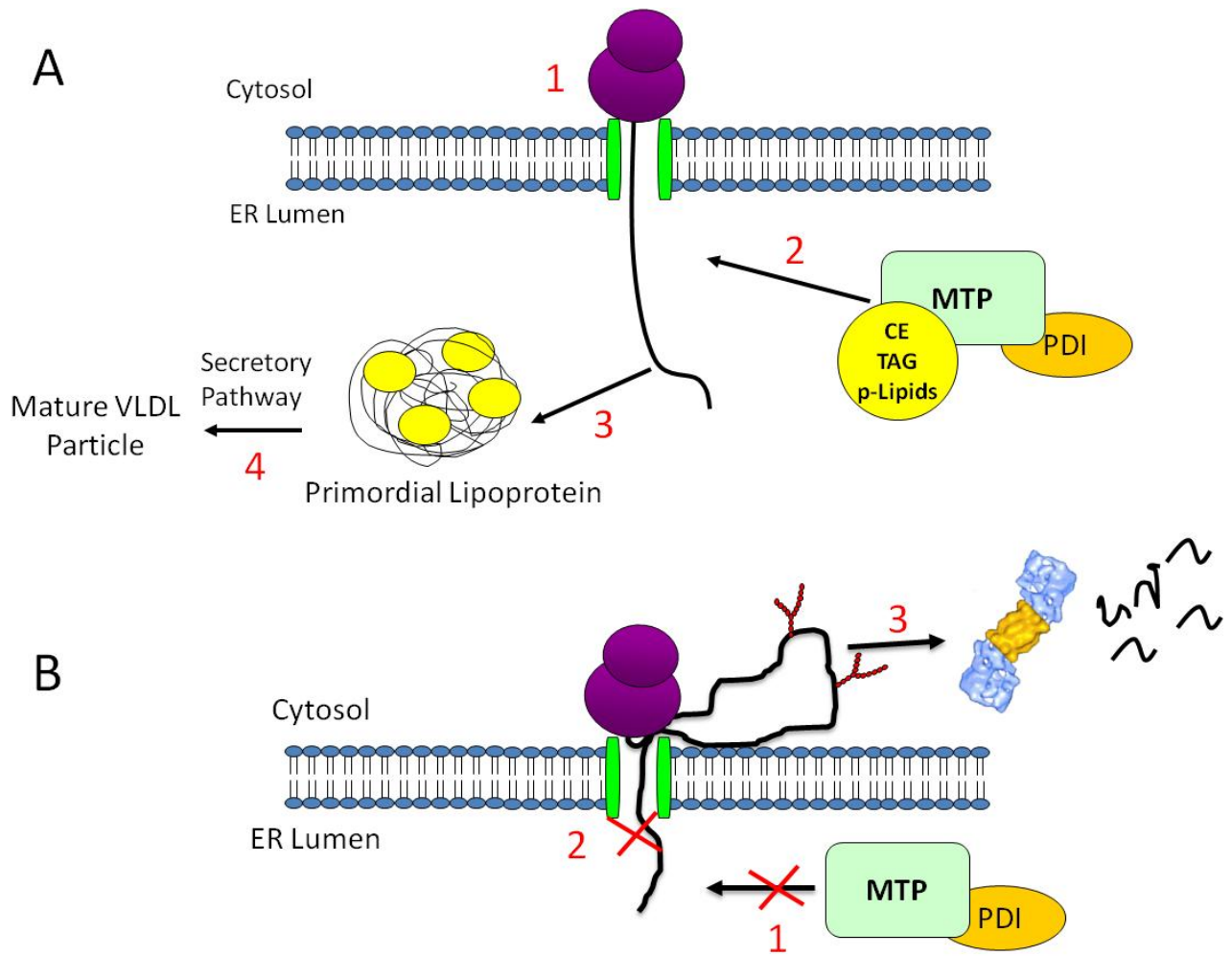
In addition to being disulfide bond rich, the  $\beta\alpha_1$  domain contains important binding sites for factors associated with the formation and metabolism of the lipoprotein particle. Amino acid residues 1-263 and 408-725 of ApoB have been reported to function as binding sites for microsomal triglyceride transfer protein (MTP) (Liang and Ginsberg, 2001; Wu et al., 1996). MTP is an ER luminal protein expressed in enterocytes and hepatocytes that is involved in the primary lipidation of ApoB as it enters the secretory pathway and forms a primordial lipoprotein. Furthermore, the first 771 amino acids of ApoB contains the LPL binding domain which is required for efficient digestion of triglycerides into fatty acids for utilization by peripheral tissues (Yang et al., 1986). Therefore the  $\beta\alpha_1$  domain, which comprises the first 1000 amino acids of ApoB is not only important for the entrance of ApoB into the secretory pathway and the formation of the primordial lipoprotein particle but it is also critical for the distribution of fatty acids to peripheral tissues by the conversion of VLDL to LDL by LPL during circulation (Hussain et al., 2000; Yamaguchi et al., 2006). Importantly, the  $\beta\alpha_1$  domain is present in the all of the ApoB constructs I utilized in my studies.

Another modification that affects the folding and amphipathicity of ApoB is the glycosylation of the protein. There are 20 putative N-linked glycosylation sites, 16 of which have been confirmed to be utilized in vivo. Interestingly, 10 of these 16 sites are located between residues 2752 and 4210, which are primarily in the  $\beta_2$  sheet region (2600-4000 residues) (Figure 1) (Harazono et al., 2005). Therefore, the majority of these glycosylation sites are absent from the isoforms examined in the majority of my experimental studies (ApoB48 and ApoB29).

Previous research has shown that the treatment of HepG2 cells (human hepatocyte cell line) with tunicamycin, a compound known to inhibit N-linked glycosylation, enhanced both proteasomal and nonproteasomal degradation of ApoB. However, they did not observe a change in the rate of the under-glycosylated ApoB secretion (Liao and Chan, 2001). Further studies were conducted on ApoB truncation mutants (ApoB17, ApoB37 and ApoB50) lacking N-link glycosylation sites. The glycosylation sites were abolished by mutating the asparagine residues to glutamines. The loss of glycosylation sites not only decreased the secretion efficiency of the mutants, but also reduced the lipid binding affinity of the ApoB37 and ApoB50 isoforms. ApoB17 is too small to have significant lipid associations and therefore was not examined (Vukmirica et al., 2002). Overall, both of these studies indicate that the presence of N-linked glycosylation residues in ApoB is important for the efficient secretion of the lipoprotein particle.

### **1.1.3 Translocation And Trafficking Of Apolipoprotein B**

The translocation of ApoB into the ER is the first committed step in the construction and secretion of a lipoprotein particle. ApoB is co-translationally targeted for translocation into the ER by a 27 amino acid signal peptide (Figure 2), which is cleaved upon entrance into the ER (Chuck and Lingappa, 1992; Chuck and Lingappa, 1993). Mutations in this signal sequence have been linked to ApoB secretion deficiency (Sturley et al., 1994). The efficiency of ApoB's translocation into the ER is dependent upon core lipid levels in the ER and the function of MTP, protein complex that transfers lipids onto ApoB to form the primordial lipoprotein, which was introduced above. MTP also functions as a protein disulfide isomerase to form disulfide bonds in the translocating polypeptide (Figure 3) (Mitchell et al., 1998). Mice that overexpress



### **Figure 3: ApoB Translocation Efficiency Depends On The Lipid Transfer Activity Of MTP**

A: 1) ApoB co-translationally translocates into the ER. 2) During its translation and translocation, lipids are loaded onto the polypeptide by MTP. In addition, disulfide bonds are formed by the PDI subunit of MTP. 3) The disulfide bond formation and lipid addition favor forward translocation of ApoB into the ER and formation of a dense primordial lipoprotein particle. 4) The primordial lipoprotein exits the ER via COPII vesicles and is fully lipidated to a VLDL particle at a later stage in the secretory pathway after which it is secreted from the hepatic cell.

B: 1) Under conditions of insufficient lipid synthesis or MTP function is blocked (by genetic mutation or MTP inhibitor), then the addition of lipids onto ApoB as it co-translationally translocates into the ER is prevented. 2) Subsequently, the translocation of ApoB into the ER stalls as translation continues. 3) ApoB then becomes a bi-topic protein in the ER translocon with amino acid residues exposed to the ER lumen and the cytoplasm. The cytoplasmically localized ApoB residues are polyubiquitinated and as a result ApoB is co-translationally targeted for degradation by the proteasome.

MTP in their hepatic tissue exhibit higher circulating VLDL levels compared with the wildtype control (Tietge et al., 1999). Additional studies linked a mutation in the MTP gene that encodes a non-functional protein in patients with hypobetalipoproteinemia (Hussain et al., 2003; Linton et al., 1993). Furthermore, cell lines treated with the MTP inhibitor, 4'-bromo 3'-methylmetaqualone, exhibit reduced ApoB secretion due to stalled translocation of ApoB (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Jamil, 1996; Liao et al., 2003; Mitchell et al., 1998). This reduced translocation efficiency results in a bitopic polypeptide that is targeted for degradation via the Ubiquitin Proteasome Pathway (UPP; see below, section 1.1.4 and section 1.2) (Figure 3) (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Fisher et al., 1997; Liao et al., 1998; Sakata and Dixon, 1999; Yeung et al., 1996). Recent clinical studies reported reduced circulating VLDL levels in familial hypercholesterolemia patients treated with the MTP inhibitor, BMS-201038, in a four week trial (Cuchel et al., 2007).

The secretion of ApoB can be stimulated by supplementing media with oleic acid (OA), a fatty acid that stimulates high levels of triglyceride synthesis in cells. The correlation between enhanced triglyceride synthesis and increased ApoB secretion efficiency has been examined in two cell lines, HepG2 (human hepatocytes) and McA-RH7777 (rat hepatocytes). Both cell lines normally produce ApoB100 but not ApoB48 due to a lack of Apobec-1 expression. However, HepG2 and McA-RH7777 cells do express MTP and the secretion of ApoB can be enhanced by supplementing the media with OA (Homan et al., 1991; Mitchell et al., 1998; Pan et al., 2002). The dependence of MTP for ApoB secretion under conditions of high triglyceride synthesis was demonstrated in a monkey kidney (COS) cell line. Because COS cells do not endogenously produce ApoB and MTP researchers transformed the cells to express both proteins. When comparing MTP expressing cells to the control cells, robust secretion of ApoB

was only observed in the OA-treated cells. This suggested that the expression of both MTP and sufficient triglyceride synthesis were required for the efficient movement of ApoB through the secretory pathway (Patel and Grundy, 1996).

As ApoB enters the ER it associates with Sec61- $\alpha$  and Sec61- $\beta$ , components of the translocon, the ER localized channel through which proteins enter the lumen of the ER (Chen et al., 1998; Mitchell et al., 1998; Pariyarath et al., 2001). Specifically, Fisher and colleagues probed ApoB-Sec61- $\alpha$  interactions under conditions of low ApoB secretion (no OA added to the media) and high ApoB secretion (OA supplemented media). A corresponding increase in the amount of ApoB associated with the translocon was detected in the cells not treated with OA. Furthermore, the authors reported that the interaction between ApoB and the translocon was stable in cells treated with puromycin and potassium acetate, a treatment known to disrupt ribosome-nascent polypeptide interactions. These data suggest that the ApoB-translocon interaction does not require the ribosome (Pariyarath et al., 2001).

Studies have also been conducted on the translocation of ApoB constructs with differential N and C terminal epitope tags in both HepG2 and chinese hamster ovary (CHO) cell lines. The CHO cell lines do not express MTP or ApoB and therefore when transiently transfected with a human ApoB expression construct, they function as a good model system for enhanced ApoB degradation. In both cell lines, proteinase K and trypsin sensitivity, and immunoprecipitation data support a model in which a stalled translocation intermediate is evident resulting in a bitopic orientation of the protein in the translocon with the N-terminus encapsulated in the ER lumen while the C-terminus is exposed to the cytoplasm (Figure 3) (Liang et al., 2000; Pariyarath et al., 2001).

While it is clear from these studies that ApoB assumes a bitopic orientation in the ER during stalled translocation, additional research sought to address the extent of the exposure of the translocated segment of ApoB to ER luminal factors. The presence of N-linked glycosylation residues, a protein modification that occurs in the ER lumen, suggests that a portion of ApoB is exposed to ER luminal factors before it is targeted for degradation (Chen et al., 1998). In addition, researchers utilized release of cells from puromycin treatment to synchronize the activity of ribosomes and subsequently observed polyubiquitination of incompletely synthesized ApoB polypeptides (Zhou et al., 1998). These data and research by others confirms that ApoB is in a bitopic orientation in the translocon where portions of the polypeptide are exposed to the environments of both the ER lumen and the cytoplasm when translocation is stalled. This includes the observation that ApoB is found associating with both ER luminal and cytoplasmic factors, such as molecular chaperones (see below, section 1.4), and is polyubiquitinated and targeted for degradation before translation of the polypeptide is complete (Chen et al., 1998; Gusarova et al., 2001; Liang et al., 1998; Liang et al., 2003; Liao et al., 1998; Zhou et al., 1995) (Figure 3).

Two additional models have been proposed to account for the inefficient translocation of ApoB into the ER. First, the Lingappa lab proposed that putative pause transfer sequences (PTS) are present in the polypeptide and contribute to the slowing of the translocation of ApoB into the ER (Chuck and Lingappa, 1992; Chuck and Lingappa, 1993; Kivlen et al., 1997). Furthermore, the Scott lab suggested that translational pausing of the ribosome on the mRNA transcript ceases the production of the polypeptide and as a result the translocation of ApoB stalls (Pease et al., 1995). However, subsequent studies by the Fisher lab showed that there was little to no post transcriptional regulation of ApoB synthesis via translational pausing or mRNA instability

(Sparks et al., 1992; Yamaguchi et al., 2006). Additional studies by the Fisher and Ginsberg labs have focused on linking the presence of beta sheet domains in ApoB to the slowed translocation observed by others (Liang et al., 1998; Yamaguchi et al., 2006). Using chimeric proteins, it was found that the addition of ApoB  $\beta_1$  domain (ApoB29-34% and ApoB36-41%) to an efficiently translocated protein, albumin, resulted in protein stalling. However, the addition of ApoB sequences lacking beta sheets but containing the putative PTS reported by Lingappa lab (ApoB12-17%) and regions with no  $\beta$  sheets and no PTS (ApoB49-54%) had no effect on the efficiency of translocation (Yamaguchi et al., 2006). These data suggest that the presence of the  $\beta_1$  sheet in ApoB and not translational pausing or PTS contribute instead to the translocation stalling. Because the  $\beta_1$  domain is proposed to be a portion of ApoB that intimately associates with the lipid core of the particle these data also correlate with the requirement for functional MTP to mediate efficient ApoB translocation.

Upon successful translocation of ApoB into the ER and addition of lipids by MTP, a primordial lipoprotein is formed that is dense in comparison to the VLDL particle it will ultimately form. The size of the primordial lipoprotein particle is ~20nm and the final mature VLDL particles is much larger at ~80-200nm (Figure 3) (Gusarova et al., 2003). Due to the relatively large size of ApoB containing particles it had been suggested that these particles may require a unique mechanism to be transported from the ER (Fromme and Schekman, 2005). However, the Fisher lab examined the mechanism by which ApoB containing particles are transported from the ER to the Golgi and determined that they exit the ER in a canonical COPII dependent manner due to the presence of Sec23, a major COPII component, in vesicles containing ApoB. In addition, they observed by indirect immunofluorescence that the ER to Golgi transport of ApoB was blocked in cells expressing a dominant negative mutant form of

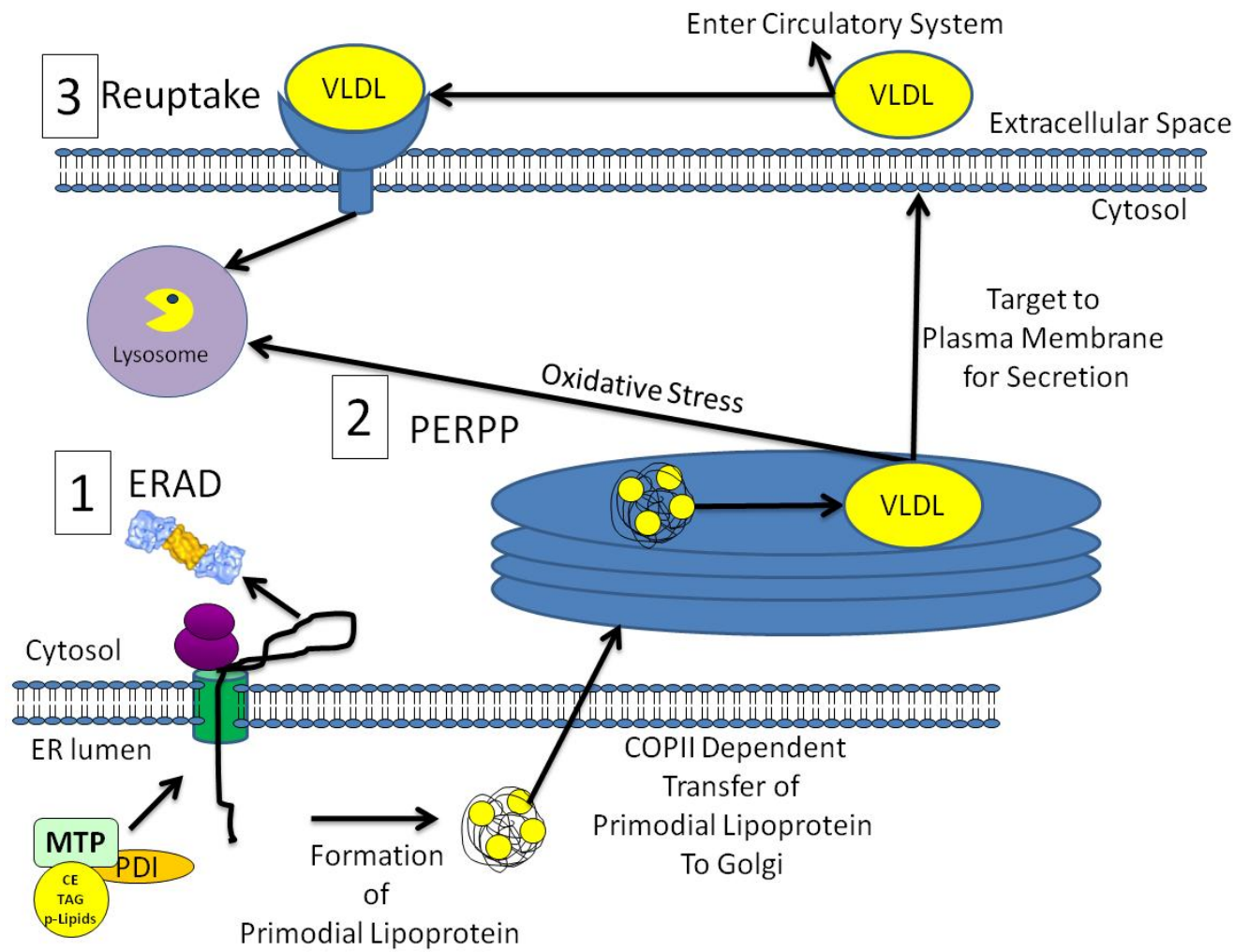


Sar1 (Gusarova et al., 2003). Sar1 is a small GTPase that is required for the recruitment of COPII components to the ER and the mutant blocks the ER to Golgi trafficking for most cargo (Kuge et al., 1994). Intriguingly, Sar1 mutants are linked to lipid absorption disorders (Jones et al., 2003).

The cellular location for final lipid addition to the primordial lipoprotein during the formation of a mature VLDL particle is also uncertain and has been proposed to be localized to both the rough-ER and post-ER compartments in the cell (Kulinski et al., 2002; Tran et al., 2002). Fractionation experiments performed in rat hepatoma cells, McA-RH7777, treated with OA to support robust triglyceride synthesis and high levels of VLDL particle secretion revealed that the fractions corresponding to ER vesicles contained a relatively dense particle compared with Golgi fractions. Only in the Golgi were VLDL particles found. These data suggest that the final lipid assembly onto ApoB to form the mature VLDL particle occurs post-ER (Gusarova et al., 2003). Overall, the entry of ApoB into the secretory pathway is tightly regulated and requires core lipidation in the ER and Golgi in order to form the mature VLDL particle.

#### **1.1.4 Apolipoprotein B Degradation**

As stated previously, ApoB is targeted for degradation by the ubiquitin proteasome pathway (UPP) as the polypeptide is being synthesized and translocated into the ER. In addition to the UPP, ApoB can also be degraded by two additional pathways, the post-ER pre-secretory proteolysis pathway (PERPP) and the re-uptake pathway. These three distinct degradation pathways within the cell allow for multiple checkpoints along the pathway of VLDL particle synthesis (Figure 4).



**Figure 4: ApoB Synthesis And Secretion Is Regulated By 3 Distinct Degradation Pathways.**

ERAD 1: ApoB is targeted to ERAD under conditions of poor translocation efficiency into the ER such as blocking MTP function which limits the addition of fatty nutrients (CE, cholesterol ester; TAG, triacylglycerol; and p-lipid, phospholipid) to the translocating polypeptide. The degradation of the bitopic protein is modulated by chaperones and components of the UPP.

PERPP 2: Following production of an ApoB containing primordial lipoprotein particle in the ER, the particle is trafficked via COPII vesicles to the Golgi compartment. In the Golgi additional core lipidation occurs and the mature VLDL particle is then targeted to the plasma membrane for secretion into the circulatory system. Under conditions of oxidative stress, the primordial lipoprotein is targeted by autophagy to the lysosome for degradation by proteases.

Reuptake 3: If the mature ApoB particle that has been secreted from the hepatic cell does not diffuse from the cell surface in it may interact with the LDL receptor and heparin sulfate proteoglycans on the cell surface that mediates endocytosis and subsequent degradation by the proteases in the lysosome.

The factors regulating the degradation of ApoB by the UPP interact with the polypeptide while it is associated with the ER. Therefore, ApoB undergoes a particular subset of UPP degradation termed ER-associated degradation (ERAD) (McCracken and Brodsky, 1996). In ERAD, the misfolded or aggregation prone protein is bound by molecular chaperones, poly-ubiquitinated, and targeted for proteasomal degradation (for a recent review see (Brodsky, 2007)). ApoB ERAD is enhanced under conditions of poor triglyceride synthesis or lipid transfer by MTP (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Jamil, 1996). ApoB degradation occurs co-translationally, is regulated by molecular chaperones, and depends upon the chymotrypsin like activity of the proteasome (Benoist and Grand-Perret, 1997; Du et al., 1999; Fisher et al., 1997; Gusarova et al., 2001; Liang et al., 2003; Liao et al., 1998; Linnik and Herscovitz, 1998; Sakata and Dixon, 1999; Tatu and Helenius, 1999; Yeung et al., 1996; Zhang and Herscovitz, 2003; Zhou et al., 1998). Typical ERAD substrates are targeted for degradation based upon their folding status, however both ApoB and another enzyme involved in VLDL synthesis, HMG CoA reductase (see below, section 1.1.5), undergo a specialized form of ERAD that is metabolically controlled. ERAD and select molecular chaperones and factors involved in the degradation of misfolded and aggregation prone proteins will be covered in the following sections. Then, I will revisit the current model for the contribution of molecular chaperones to the synthesis and degradation of ApoB.

PERPP is a proteolytic pathway that occurs after the MTP lipidation step, but before secretion of the lipoprotein particle. The pathway targets lipid-rich ApoB molecules for degradation and occurs before the particle is secreted from the cell (Fisher et al., 2001). PERPP can be stimulated by the metabolism of omega-3 fatty acids due to oxidative stress caused by the metabolism of these fatty acids (Pan et al., 2004). The post-ER degradation of ApoB by PERPP

is nonproteasomal (Cardozo et al., 2002). Recently, the Fisher laboratory proposed that ApoB is targeted from the Golgi to autophagosomes during oxidative stress. Aggregates of ApoB form in the presence of oxidative stressors, such as linolenic acid, a fatty acid associated with low plasma triglyceride levels (Djoussé et al., 2003). The formation of these aggregates could be blocked by treating cells with brefeldin A and nocodazole, inhibitors of ER to Golgi trafficking, and monensin, an inhibitor of protein exit from the Golgi. The ApoB aggregates were stabilized in the presence of 3-methyladenine, a compound which blocks autophagosome formation; E64d, an inhibitor of lysosomal/endosomal cathepsins; and siRNA treatment against Atg7, an autophagy-requiring gene (E.A. Fisher, manuscript submitted to PNAS, 2008). The new data suggest that the metabolism of omega-3 fatty acids in hepatic cells causes oxidative stress, thus inducing a cellular response that targets ApoB to autophagosomes for degradation and reducing the amount of secreted VLDL particles. These data also correlate well with studies that indicate that diets high in omega-3 fatty acids correspond with a reduced risk for developing CAD (Williams and Fisher, 2005).

The third pathway is the reuptake pathway and it occurs after the VLDL particle has been secreted from the hepatocyte, but before it diffuses away to enter the circulatory system. The particle can be recognized by the LDL receptor or heparan sulfate proteoglycans. The nascent particle is then endocytosed and subsequently degraded (Sorci-Thomas et al., 1992; Twisk et al., 2000; Williams et al., 1992). Sterol deprivation and treatment of cells with omega-3 fatty acids and isoflavones have also been shown to upregulate the re-uptake pathway by inducing the expression of additional LDL receptors. This increases the chance of an interaction between a newly secreted particle and a receptor before the particle has had time to diffuse (Borradaile et al., 2002). The treatment of cells with omega-3 fatty acids also increases the amount of

apolipoprotein E (ApoE) expressed in the cells with the ApoB. ApoE is a transferrable lipoprotein that contributes to the receptor mediated endocytosis of ApoB into hepatocytes (Borradaile et al., 2002; Gusarova et al., 2007). Overall, ERAD, PERPP and re-uptake cumulatively regulate the synthesis of VLDL particles based on the metabolic status of the cell.

### **1.1.5 HMG-CoA Reductase**

The ER localized polytopic membrane protein 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is a rate-limiting enzyme in the mevalonate pathway by which cholesterol and other essential isoprenoids are produced. HMG-CoA reductase production increases when the uptake of circulating cholesterol is limited, thus increasing the endogenous synthesis of cholesterol by the hepatocyte (Stange et al., 1981). Like ApoB, the synthesis of HMG-CoA reductase is tightly regulated by several pathways based on the metabolic status of the cell, including transcriptional regulation and ERAD (McGee et al., 1996; Ravid et al., 2000). The activity of HMG-CoA reductase is slowed when cells are treated with statins, a class of drugs used to treat patients with high cholesterol (Endo et al., 1976). Statin treated patients exhibit a corresponding drop in VLDL particle production due to reduced endogenous cholesterol synthesis. These data indicate that inhibition of lipoprotein particle assembly can be used to treat patients with high circulating levels of cholesterol (Mabuchi et al., 1981). However, statins are not tolerated well by all patients and common small nucleotide polymorphisms of the HMG-CoA reductase gene are statin insensitive (Chasman et al., 2004). Therefore, additional therapies targeting lipoprotein synthesis and secretion are needed. Although the technique has not been adapted for humans, a liver targeted ApoB siRNA can reduce circulating levels of VLDL particles in mice and nonhuman primates (Soutschek et al., 2004; Zimmermann et al.,

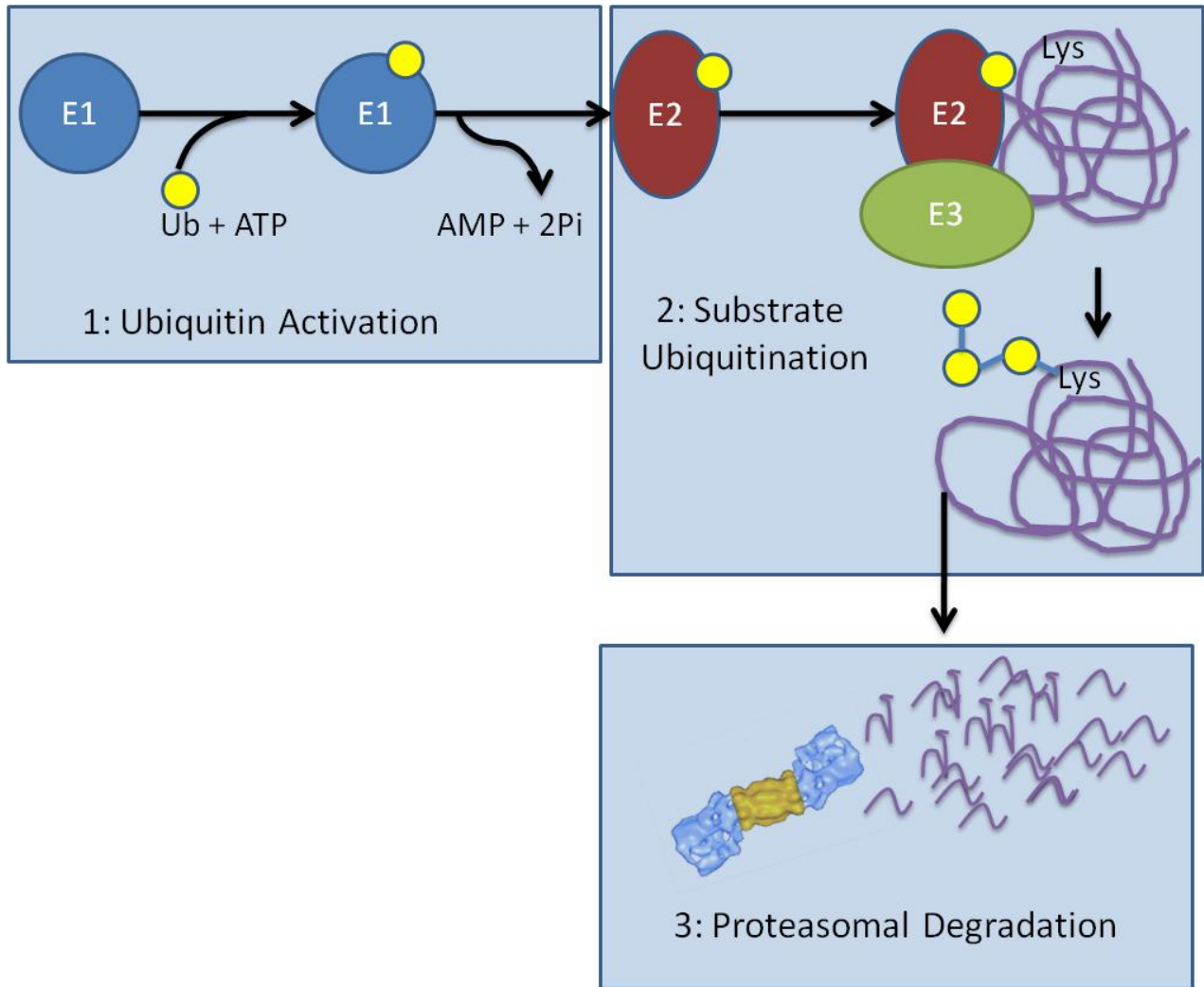
2006). Therefore, decreasing ApoB synthesis may be a viable target for reducing lipoprotein particle secretion and preventing the development of CAD.

## **1.2 UBIQUITIN PROTEASOME PATHWAY**

The ubiquitin proteasome pathway (UPP) is a process by which proteins are selected, marked for degradation by multiple additions of a protein conjugate called ubiquitin, and degraded by the proteasome (Figure 5). The UPP is not only involved in ER quality control via ERAD (see below), but is also involved in immune system function, cell cycle control and the regulated turnover of nuclear proteins during the cell cycle and the removal of misfolded cytoplasmic proteins. The following section will discuss the factors involved in each of these steps in the UPP: The addition of ubiquitin to protein substrates, the recognition of the polyubiquitinated substrates, and the degradation of polyubiquitinated substrates by the proteasome (For a recent review see (Meusser et al., 2005)).

### **1.2.1 ER-associated Degradation**

As noted above, ApoB undergoes a particular subset of the UPP, called endoplasmic reticulum associated degradation (ERAD) (reviewed in (Brodsky, 1996)). Protein biogenesis and folding is a complex process in which proteins must achieve their proper conformations. For secreted proteins, these factors must also successfully travel through the secretory pathway to their site of function. This process can yield aberrant, nonfunctional polypeptides that may be held in the





### **Figure 5: The Ubiquitin Proteasome Pathway**

The Ubiquitin Proteasome Pathway is a series of enzymatic reactions that mediate the transfer of activated ubiquitin onto a target substrate.

1: Ubiquitin is activated by an E1 enzyme in an ATP dependent manner. The E1 then transfers the ubiquitin moiety onto an E2 enzyme.

2: The E2 enzyme cooperates with an E3 enzyme in the recognition of target polypeptides and the transfer of the ubiquitin moiety onto a lysine residue or the N-terminus of the substrate. The process is repeated, resulting in a polyubiquitin chain that targets the protein for degradation by the 26S proteasome.

3: The 19S cap of the proteasome contains enzymes that recognize the polyubiquitin motif and deubiquitinating enzymes that remove the polyubiquitin tag before the protein is fed into the 20S proteolytic core by the AAA ATPases in the 19S cap. The ubiquitin is then free to be activated by an E1 and enter the UPP pathway again. Shown are resulting protein fragments after degradation (Figure modified from (Meusser et al., 2005)).

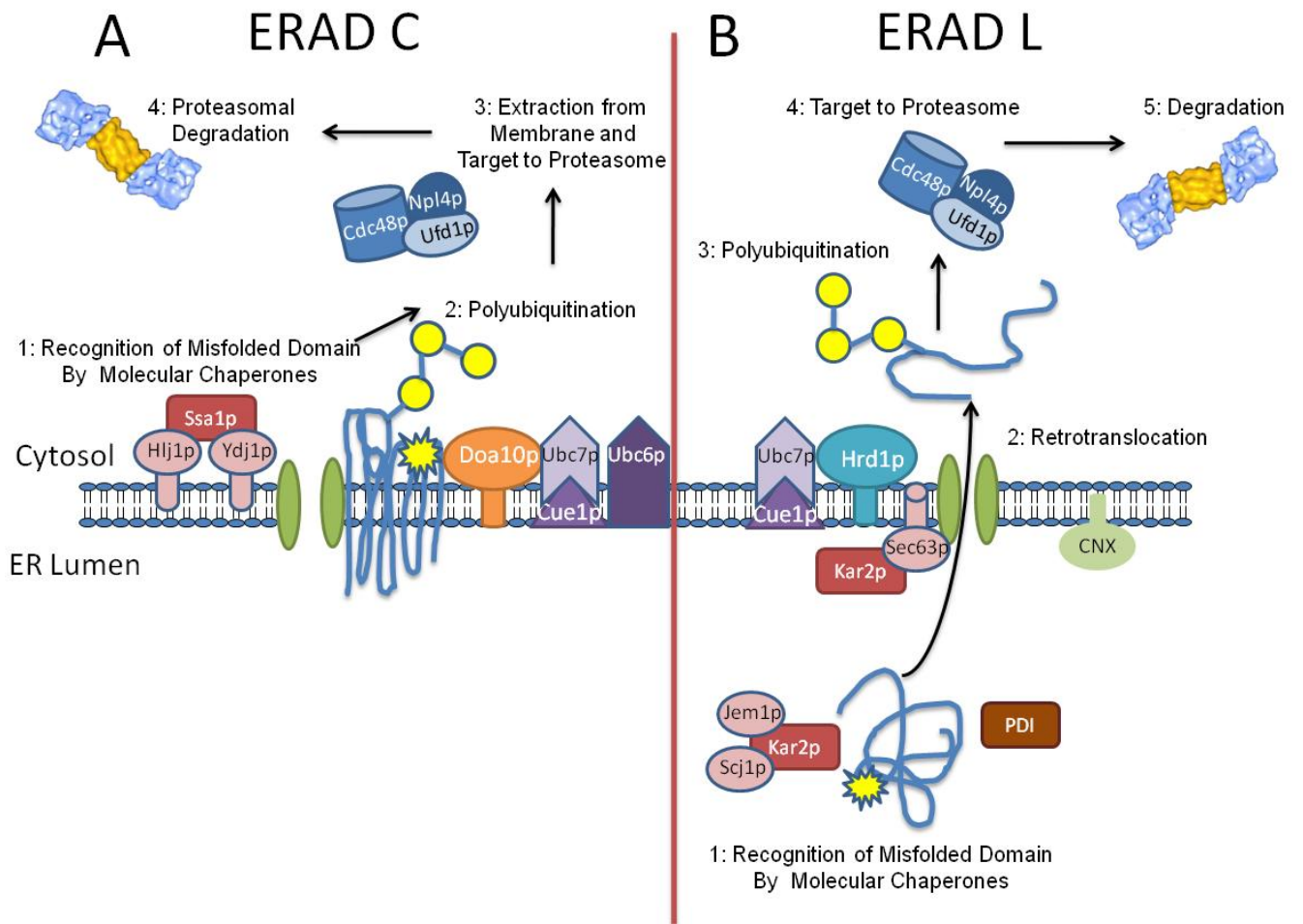
ER. The accumulation and aggregation of these misfolded proteins can cause cellular stress and even induce cell death (Ng et al., 2000; Travers et al., 2000). In ERAD, misfolded or aggregation prone proteins are targeted to the cytoplasm to be degraded by the UPP (Hampton and Bhakta, 1997; Jensen et al., 1995b; Ward et al., 1995; Zhou et al., 1998). Some ERAD substrates are retained in the ER, however others recycle from the Golgi before being retrotranslocated from the ER to the cytoplasm (Vashist and Ng, 2004). Interestingly, a few ERAD substrates are not polyubiquitinated but are still degraded by the proteasome (McGee et al., 1996; Wahlman et al., 2007; Werner et al., 1996; Yu et al., 1997). The specialized ubiquitin independent degradation of protein substrates occurs when the proteins are highly oxidized and misfolded and is a special form of post-translational processing for a few protein substrates (Rape and Jentsch, 2002; Shringarpure et al., 2003). However, the majority of protein substrates targeted by quality control pathways to proteasomal degradation utilize the UPP.

The Ng lab initially determined that there are two main varieties of ERAD, ERAD-C and ERAD-L, which require distinct components of the UPP and select molecular chaperones for their degradation. The determining factor that separates substrates into each pathway is the location of the misfolded domain. Proteins with ER lumenal misfolded domains (both soluble and membrane) are targeted to ERAD-L while proteins with cytoplasmic misfolded domains are recognized by the ERAD-C pathway (Figure 6) (Vashist and Ng, 2004). Subsequently, ERAD-M was identified as another distinct pathway for membrane proteins with disrupted transmembrane segments. ERAD-M is the least defined of the ERAD pathways. Both ERAD-L and ERAD-M require similar UPP and chaperone components, the complexes are not completely identical. In fact, ERAD-M substrate degradation has been shown to be independent of select members of the ERAD-L complex (Carvalho et al., 2006; Denic et al., 2006).

The Cdc48p (p97 in mammalian cells) complex has been shown to be important for both ERAD-C and ERAD-L. The complex in yeast consists of three factors: Cdc48p, Ufd1p, and Npl4p (Bays et al., 2001b; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). The AAA (ATPases Associated with diverse cellular Activities) ATPase activity of Cdc48p drives the extraction of polyubiquitinated membrane proteins for proteasomal degradation (Jentsch and Rumpf, 2007; Nakatsukasa et al., 2008; Ye et al., 2003). In addition, the complex is important for the retrotranslocation and targeting of soluble ERAD-C substrates to the proteasome for degradation (Nakatsukasa et al., 2008; Rabinovich et al., 2002; Richly et al., 2005; Ye et al., 2001; Ye et al., 2003).

Unlike the Cdc48p complex, the molecular chaperone requirements for substrate recognition and targeting to degradation are distinct for the ERAD-L and ERAD-C pathways. Molecular chaperones are a class of proteins that assist in the folding of protein substrates to their proper conformation. Aberrantly folded proteins are targeted to the cytosol to be degraded by the proteasome through the action of substrate specific molecular chaperones and UPP components. (See below) (Table 1) (For a recent review see (Nishikawa et al., 2005)).

As it is estimated that approximately 30% of all newly synthesized proteins travel the secretory pathway and that protein folding is a complex process it is not surprising that ERAD is linked to multiple human diseases (Ghaemmighami et al., 2003; Lukacs et al., 1994; Thomas et al., 1995)(for a recent review see (Brodsky, 2007)). For example, the disease cystic fibrosis is caused by a mutation in the gene encoding the CFTR chloride channel. Although the function of the protein is not greatly altered, the folding of the channel is inefficient (Jiang et al., 1998; Lukacs et al., 1994). As a result CFTR is turned over rapidly by ERAD and very little protein



### **Figure 6: ERAD-C And ERAD-L**

A: ERAD-C Membrane proteins with misfolded cytosolic domains (indicated by the star) are recognized by molecular chaperones (1), Hsp70 (Ssa1p) and Hsp40 (Hlj1p, and Ydj1p), and targeted for polyubiquitination (depicted as yellow circles) (2). The polyubiquitination is mediated by the E2s, Ubc7p and Ubc6p, and the E3, Doa10p. While Ubc6p is a membrane protein, Ubc7p is anchored at the ER membrane by the protein Cue1p and this interaction is essential for the function of Ubc7p in ERAD. The extraction of the protein from the membrane (3) requires the activity of the Cdc48p complex, which is a heterocomplex comprised of Cdc48p, Npl4p, and Ufd1p. The substrate is subsequently degraded by the 26S proteasome (4).

B: ERAD-L Soluble proteins that are translocated into the ER with misfolded domains (indicated by the star) or membrane proteins with misfolded luminal domains (not shown) are recognized by the ER luminal chaperones (1): Hsp70 (Kar2p), Hsp40s (Scj1p, Jem1p, Sec63p), and in some cases calnexin (CNX), and protein disulfide isomerase (PDI). These proteins assist with targeting the polypeptide for retrotranslocation (2) into the cytosol where the substrate is polyubiquitinated (3) by the E2, Ubc7p and the E3, Hrd1p. The polyubiquitinated protein is subsequently targeted to the proteasome by the Cdc48p complex (4) for degradation (5) (Figure modified from (Nishikawa et al., 2005)).

For simplicity ERAD-M is not shown in this figure.

**Table 1: Select UPP Factors And Molecular Chaperones Discussed In This Document**

<b>Protein Family</b>	<b>Cellular Function</b>	<b>Specific Members Discussed:</b>
E2 Ubiquitin Conjugating Enzymes	Carrier of activated ubiquitin	Yeast: Ubc6p, Ubc7p Mammalian: Ubc7
E3 Ubiquitin Ligase	Mediate transfer of ubiquitin to target substrate	Yeast: Doa10p, Hrd1p Mammalian: gp78
Cdc48p Complex	Heteroprotein complex containing a AAA ATPase involved in membrane protein extraction and targeting polyubiquitinated substrates for proteasomal degradation	Yeast: Cdc48p, Ufd1p, Npl4p Mammalian: p97, Ufd1, Npl4
Hsp70	Chaperone involved in translocation, protein folding and ERAD	Yeast: Ssa1p, Ssb1p, Kar2p Mammalian: Hsp70, BiP
Hsp40	J domain containing chaperone that stimulates Hsp70 ATPase activity	Yeast: Ydj1p, Hlj1p, Scj1p, Jem1p
Hsp70 NEF	Nucleotide exchange factors for Hsp70	Yeast: Snl1p, Fes1p, Sse1p
Hsp90	Chaperone involved in folding a diverse set of client proteins and ERAD	Yeast: Hsp82p Mammalian :Hsp90
Hsp110	Chaperone with holdase activity and functions as a NEF for Hsp70s	Yeast: Sse1p Mammalian: Hsp110
Lectin	ER luminal chaperone that monitors folding of monoglycosylated substrate proteins	Yeast: Calnexin Mammalian: Calnexin and Calreticulin
Protein Disulfide Isomerase	ER luminal chaperone that mediates proper disulfide bond formation in proteins	Yeast: Pdi1p, Eug1p, Mpd1p, Mpd2p, Eps1p Mammalian: PDI1
p58 <sup>IPK</sup>	ER luminal J domain containing protein involved in translocation	Mammalian: p58 <sup>IPK</sup>

escapes the secretory pathway to function at the cell surface (Cheng et al., 1990; Jensen et al., 1995b; Ward et al., 1995). As mentioned previously, both ApoB and HMG-CoA reductase undergo a specialized form of ERAD that is metabolically controlled. Importantly, ERAD is a highly conserved process with similar factors and chaperones regulating the pathway in both yeast and mammals and therefore studies on ERAD in model systems have helped to define the ERAD pathway in higher organisms. Select ERAD substrates and their chaperone and UPP component requirements are listed in Table 2.

### **1.2.2 Polyubiquitination**

The addition of the 76 amino acid conserved polypeptide ubiquitin to cellular proteins regulates a range of eukaryotic cellular functions such as endocytosis, transcription, and targeting proteins for proteasomal degradation. A polyubiquitin chain is formed by the successive addition of ubiquitin to specific lysine residues or the N-terminus of previously attached ubiquitin moieties. The specific lysine residue that is used for ubiquitin conjugation to form the polyubiquitin chain corresponds to the ultimate substrate target. The two best characterized linkages are Lys-48 and Lys-63. Lys-48 polyubiquitin chains efficiently target substrates to the proteasome for degradation while Lys-63 is involved in DNA repair and signal transduction (Kerscher et al., 2006). The addition of ubiquitin to the target polypeptide is mediated by the sequential actions of three enzymes: E1, an activating enzyme that forms a thiol ester with the carboxyl group of G76, thereby activating the C-terminus of ubiquitin for nucleophilic attack; E2, a conjugating enzyme that transiently carries the activated ubiquitin as a thiol ester; and E3, a ligase that

**Table 2: Select ERAD Substrates And Their UPP And Chaperone Requirements For ERAD**

Substrate	Soluble or Membrane	Description	Factors involved in ERAD discussed:
CPY*	S	A point mutant of carboxypeptidase Y (CPY) that is misfolded	Ubc7p, Hrd1p, Kar2p, Scj1p, Jem1p
$\Delta$ ssCPY*	S	CPY* protein lacking the signal sequence therefore mislocalized to the cytoplasm	Ssa1p, Ydj1p
CT*	M	ER Luminal CPY* with single TM segment	Ubc7p, Hrd1p
AT-Z	S	Anti-trypsin Z variant that is aggregation prone and degraded by ERAD	Kar2p
Sec61-2	M	Mutant form of Sec61, the major protein of the translocation channel	Ubc6p, Ubc7p, Hrd1p
CFTR	M	Polytopic membrane protein that is an ABC transporter and functions as a chloride channel	Ubc6p, Ubc7p, Doa10p, Ssa1p, Hsp90p, Aha1p
Ste6p*	M	C-terminal truncation mutant of polytopic membrane protein that is an ABC transporter	Ubc6p, Ubc7p, Doa10p, Ssa1p, Ydj1p, Hlj1p
HMG CoA R or HMG2p	M	Polytopic membrane protein involved in cholesterol biosynthesis and whose ERAD is metabolically controlled in mammalian cells	Ubc7p, Hrd1p
VHL	S	von Hippel-Lindau (VHL) tumor-suppressor protein that undergoes chaperone-mediated folding	Ssa1p, Sse1p, Hsp90p, Sti1p
Dio2	M	Human deiodinase D2 (Dio2) has its N-terminal end inserted in the ER membrane and a cytosolically exposed c-terminal domain	Ubc6p, Ubc7p
Mat $\alpha$ 2 repressor	S	Transcriptional repressor of alpha mating type gene expression	Ubc6p, Ubc7p, Doa10p
Mutant Insulin Receptors	M	Mutants Insulin Receptor that do not fold properly	Hsp90p
$\Delta$ g pp $\alpha$ f	S	Pre-pro-alpha factor that lacks its glycosylation sites	Kar2p Scj1p, Jem1p



transfers the ubiquitin from the E2 to the lysine residue or N-terminus on the target polypeptide (Figure 5) (Hershko et al., 1983) (For a recent review see (Hochstrasser, 2006)).

E1s are essential proteins that are responsible for activating the ubiquitin for a multitude of cellular purposes (McGrath et al., 1991). Yeast and humans express a limited number of E2s and a larger number of E3s. The combination of E2 and E3 interactions determines the specificity of the protein complex for substrate recognition. For example, the yeast E2s Ubc6p and Ubc7p are both reported to cooperate with the E3, Doa10p, while only Ubc7p interacts with the E3, Hrd1p (Hampton, 2002; Hirsch et al., 2004; Ravid et al., 2006). The function of these specific E2s and E3s will be described in detail in the following sections. Overall, the graded distribution of one E1 to a few E2s to many E3s allows for the allotment of ubiquitin among different E2/E3 pairs and as a result only specific polypeptide substrates and cellular processes are targeted (For modification reviews see (Hershko and Ciechanover, 1998; Kerscher et al., 2006)).

#### **1.2.2.1 E2 Ubiquitin Conjugating Enzymes**

The ubiquitin conjugating enzymes, E2s, function as carriers of activated ubiquitin to a partner E3 that will assist with the transfer of the activated ubiquitin to a specific target substrate. E2s share a conserved core of ~150 amino acids with N or C-terminal extensions that mediate the specific interactions with their cognate E3s (Jensen et al., 1995a). In *S. cerevisiae*, there are two primary E2s that are linked to the ERAD of misfolded protein substrates, Ubc6p and Ubc7p. The ER localized tail-anchored integral membrane protein, Ubc6p, was the first yeast E2 whose involvement in ERAD was demonstrated (Sommer and Jentsch, 1993). The ubiquitin conjugating domain is located at the N-terminus and the C-terminus is a stretch of hydrophobic amino acids that function as a transmembrane domain to tether Ubc6p to the ER membrane. The

localization of Ubc6p to the ER membrane is essential for its function in ERAD (Sommer and Jentsch, 1993 and Chen 1993). Ubc6p is required for the ERAD of a mutant allele of the transmembrane protein Sec61p, Sec61-2; the membrane associated protein Dio2; and the soluble cytoplasmic protein Mat $\alpha$  2 repressor (Biederer et al., 1996; Chen et al., 1993; Ravid et al., 2006; Sommer and Jentsch, 1993).

The other E2 primarily involved in ERAD, Ubc7p, is localized to the ER membrane through its interaction with its membrane bound receptor Cue1p (Biederer et al., 1997). As stated previously, Ubc6p and Ubc7p are reported to function in the ERAD-C pathway. However, Ubc7p, but not Ubc6p, is required for the degradation of ERAD-L substrates (Vashist and Ng, 2004). For example, the known Ubc6p substrates, Dio2, Sec61-2, and Mat $\alpha$ 2 repressor, are also ubiquitinated by Ubc7p (Biederer et al., 1996; Chen et al., 1993; Ravid et al., 2006). While Ubc7p, but not Ubc6p, is responsible for the ERAD of a mutant form of carboxy peptidase y (CPY\*, a soluble protein) and Hmg2p (a multi-pass ER membrane protein) (Table 2) (Bays et al., 2001a; Hampton and Bhakta, 1997; Hiller et al., 1996). The Ng lab has correlated the function of these E2s in the separate ERAD-C and ERAD-L pathways to the cooperation of the enzymes with different cognate E3s (Figure 6). The function of the E3s and the separation of the ERAD-L and ERAD-C pathways will be discussed in the next section.

#### **1.2.2.2 E3 Ubiquitin Ligases**

The ubiquitin protein ligases, E3s, interact directly or indirectly with their protein substrate and mediate the transfer of ubiquitin from the E2 ubiquitin conjugating enzymes to the target polypeptide. There are two major classes of E3s: homologous to E6-AP C terminus (HECT) domain E3s and really interesting new gene (RING) finger domain E3s. HECT domain E3s

accept ubiquitin from the E2 and form thiol-ester intermediates with ubiquitin before transferring the ubiquitin moiety to the target substrate (Hershko and Ciechanover, 1998). The ~350 residue region contains a highly conserved cysteine residue that mediates the essential thiol ester intermediate during catalysis (Huibregtse et al., 1995). RING finger E3s bind the E2 and mediate the direct transfer of ubiquitin from the E2 to the substrate. The RING finger domain is ~70 residues and contains a set of cysteine and histidine residues that function as the ligands of two zinc ions that stabilize a characteristic globular structure. The zinc ions are catalytically inert and it is thought that the RING fingers function as molecular scaffolds to bring other proteins together in a specific and catalytically productive manner (Borden, 2000; Lorick et al., 1999). The only E3 that impacts ApoB degradation in mammalian cells is of the RING finger subset (gp78, see below (Liang et al., 2003)).

The E3s that have been shown to be important for ERAD in yeast are the ER localized proteins, Hrd1p and Doa10p. Hrd1p was discovered in a screen for proteins required for the degradation of the yeast HMG-CoA reductase, Hmg2p (Hampton et al., 1996). Subsequently, Hrd1p has been identified as the E3 for the ERAD-L pathway which targets misfolded soluble ER luminal proteins and membrane bound proteins with ER luminal misfolded domains (Bordallo et al., 1998; Hampton et al., 1996; Vashist and Ng, 2004). As state above, the only E2 currently linked to ERAD-L, and thus Hrd1p function in this pathway, is Ubc7p (Figure 6) (Bays et al., 2001a; Friedlander et al., 2000; Vashist and Ng, 2004). The RING finger domain of Hrd1p is cytosolically exposed and required for both an interaction with Ubc7p and the polyubiquitination of ERAD-L substrates (Deak and Wolf, 2001).

In contrast, substrates of Doa10p are integral membrane proteins with large cytoplasmic misfolded domains and cytoplasmic proteins (Swanson et al., 2001; Vashist and Ng, 2004). This

E3 interacts with both Ubc6p and Ubc7p in the ERAD-C pathway (Figure 6). Mutational analysis revealed that the N-terminal RING finger domain is required for the ubiquitination and degradation of its target substrates (Swanson et al., 2001). Interestingly, ERAD substrates with misfolded domains exposed to both the ER lumen and cytoplasm utilize ERAD-C, suggesting that this pathway is upstream of ERAD-L (Vashist and Ng, 2004). Recently it was reported that Hrd1p can also polyubiquitinate membrane substrates with cytosolic lesions in cells lacking Doa10p, suggesting that there is some redundancy in the polyubiquitination machinery (Figure 6) (Arteaga et al., 2006; Hoyer et al., 2004; Kota et al., 2007; Nakatsukasa et al., 2008).

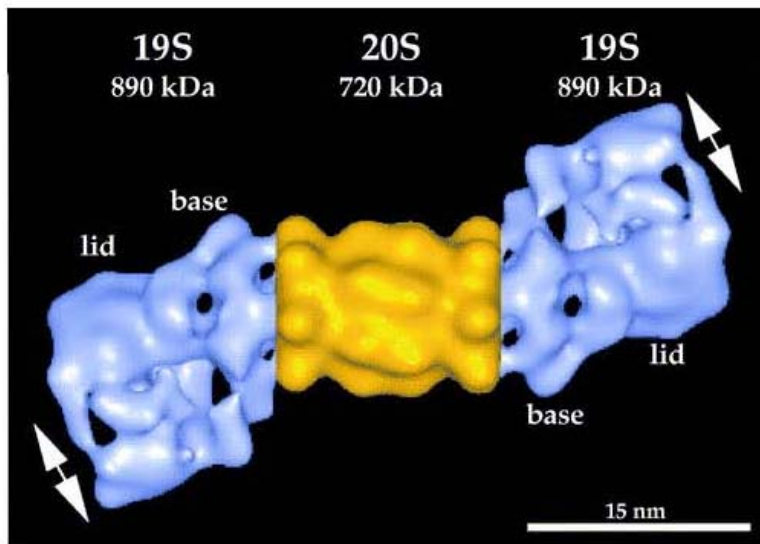
The mammalian RING finger E3, gp78, has been shown to contribute to the regulation of ApoB secretion from hepatic cells (Liang et al., 2003). gp78 was initially identified as a membrane glycoprotein in murine melanoma cells and proposed to be involved in cell migration. Subsequent research identified gp78 as the first mammalian ER-resident E3 and linked the protein to the degradation of the ERAD substrate T-cell antigen receptor CD3  $\delta$ -subunit (Fang et al., 2001). Indirect immunofluorescence and immunoprecipitation data indicate that gp78 colocalizes and associates with the mammalian Ubc7 E2 homolog. In addition, both gp78 and the yeast E3, Hrd1, exhibited ubiquitination activity in the presence of mammalian Ubc7, but not Ubc6. Overexpression of gp78 in HepG2 cells increased the polyubiquitination and proteasomal degradation of ApoB which corresponded to a decrease in the secretion of the lipoprotein. This decrease in secretion by the overexpression of gp78 could be rescued by the treatment of cells with OA (the fatty acid that stimulates ApoB secretion, see above), however the secretion of ApoB was greater in similar experiments with cells overexpressing a cytosolic E3, Itch, that does not contribute to the polyubiquitination of ApoB (Liang et al., 2003). The

identity of other ER-associated or cytosolic E3s that contribute to ApoB ERAD is a current area of investigation.

### **1.2.3 The Proteasome**

The final step in the UPP is recognition of the polyubiquitinated substrate and subsequent proteasomal degradation (Figure 5). In vitro data suggest that the 26S proteasome can directly recognize polyubiquitinated proteins and degrade them (Deveraux et al., 1994; Lipson et al., 2008; Ugai et al., 1993). In addition, the 19S cap of the proteasome is sufficient to drive the dislocation of mutant pro- $\alpha$  factor from yeast microsomes in vitro, however this substrate is not polyubiquitinated (Lee et al., 2004). These data suggest that the proteasome also is actively involved in the recognition and degradation of non-ubiquitinated substrates. As mentioned previously, additional data indicates that the proteasome requires specific escort factors such as the Cdc48p complex (the p97 complex in mammalian cells) for the presentation of ubiquitinated substrates for degradation (Figure 6) (Bays et al., 2001b; Hoyer et al., 2004; Lipson et al., 2008).

After the polyubiquitinated substrate is targeted to the proteasome by the escort factors the protein is subsequently deubiquitinated and degraded by the proteasome in an ATP dependent manner (Gottesman et al., 1997). A fully assembled 26S proteasome consists of a 20S core chamber with two 19S caps (Figure 7). The 20S subunit is a cylindrical stack of 4 heptameric rings. The catalytic sites required for proteolysis are localized on the interior of the inner rings of the 20S subunit and are only accessible through narrow pores at either end of the complex (Groll et al., 2000). The 19S cap functions as the regulatory complex and is comprised of a base and a lid. The base contains 6 ATPase units that are involved in substrate unfolding



**Figure 7: The Structure Of The 26S Proteasome**

The fully assembled 26S proteasome is comprised of two 19S caps and a 20S proteolytic core. The 19S cap contains a base and a lid comprised of at least 19 subunits (Verma et al., 2000). The lid is involved in the recognition of polyubiquitinated substrates and the deubiquitination of substrates. The base contains all 6 AAA ATPases that are involved in feeding the protein substrate into the 20S core. The 20S core is comprised of 7 different alpha subunits and 7 different beta subunits that are arranged in a stack of four seven-membered rings in an order of alpha ring, beta ring, beta ring, alpha ring. The beta rings contain the proteolytic active sites and the alpha rings are important in regulating the activation of the 20S core (Zwickl et al., 1999) (Figure from (Voges et al., 1999)).

while the lid recognizes and removes polyubiquitin from the substrate (Braun et al., 1999; Glickman et al., 1998a; Glickman et al., 1998b; Rubin et al., 1998).

Two primary models have been proposed for the mechanism by which the proteasome cleaves the polypeptide substrate into short peptides. In the “end first” model the polypeptide chain is degraded from one end and is stalled by a stop transfer signal that prevents further degradation (Orian et al., 1999; Palombella et al., 1994). The “alternative loop” model predicts that the structure folds into a hairpin loop that is threaded into the proteolytic chamber, thus allowing the proteasome to cleave an internal site (Hoppe et al., 2000; Hoppe et al., 2001; Piwko and Jentsch, 2006). The complete degradation of the substrate is blocked by the presence of tightly folded domains, the tight binding to a partner protein, and by folded domains that are near a low complexity amino acid sequence (Lee et al., 2001; Liu et al., 2003; Rape et al., 2001; Rape and Jentsch, 2002). Recent studies by Piwko and Jentsch on substrate degradation *in vivo* confirmed that the proteasome can cleave a substrate at an internal site and subsequently process the peptide in both the N and C-terminal directions. The authors proposed a model in which the proteasome cleaves the target substrate at the most flexible domains of the polypeptide, and this site can be located internally or at the N or C-terminal ends of the protein (Piwko and Jentsch, 2006).

### **1.3 MOLECULAR CHAPERONES**

Molecular chaperones are a class of proteins that are involved in a variety of processes during protein biogenesis, including protein translocation, helping polypeptides fold into their native conformation, preventing protein aggregation, catalyzing post-translational modifications, and

targeting misfolded substrates to the UPP for degradation. The three main classes of chaperones are: heat shock proteins (*e.g.*, Hsp70, Hsp40, Hsp110, and Hsp90); ER lectins (*e.g.*, calnexin); and thiol oxidoreductases (*e.g.*, PDI). The roles of these chaperones and their co-factors involved in protein biogenesis and ERAD will be discussed in the following sections (For a recent review see (Buck et al., 2007))

### **1.3.1 Structure and Function of Select Cytosolic Molecular Chaperones**

Five types of cytosolic molecular chaperones and their co-factors will be discussed in this section. For a complete list of the chaperones and co-factors covered see Table 1. The ERAD substrates and the chaperones that regulate their degradation that are discussed in the following sections are listed in Table 2.

#### **1.3.1.1 Hsp70**

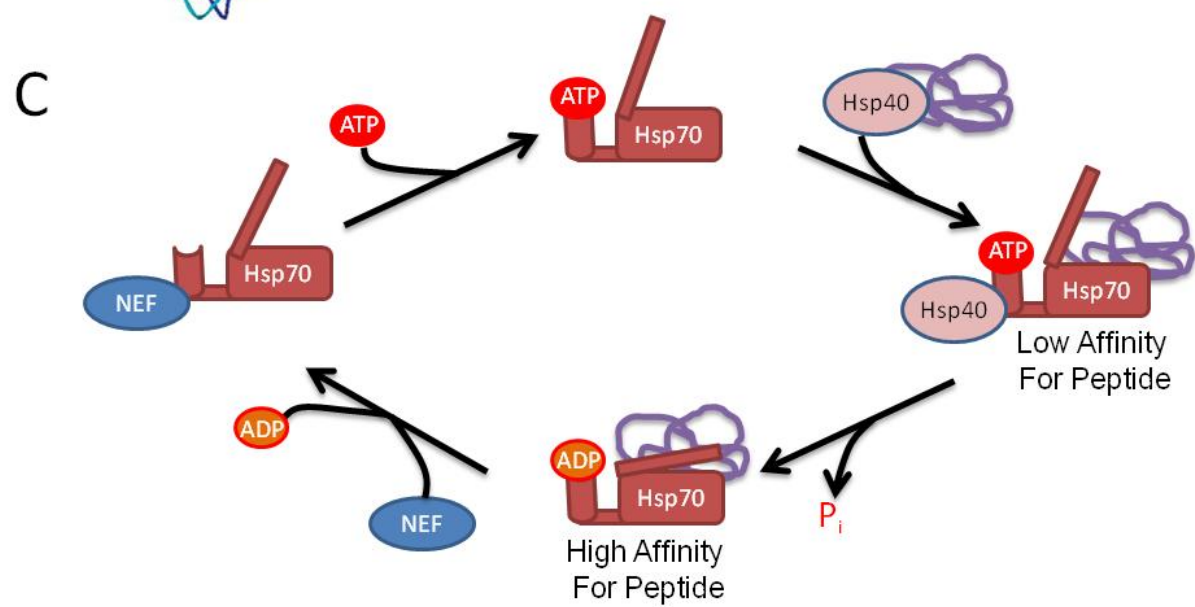
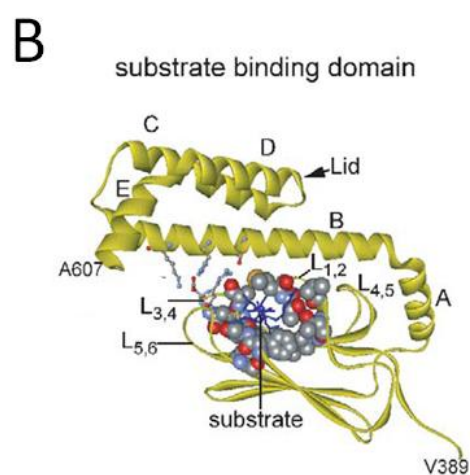
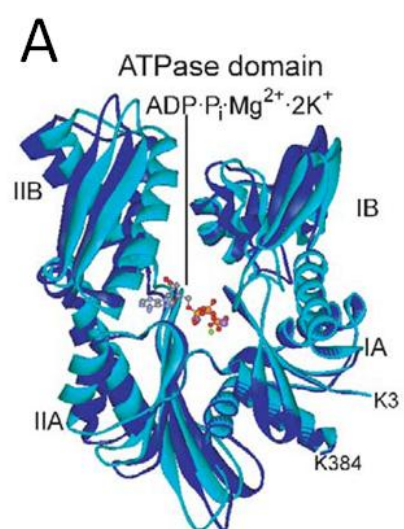
Hsp70s are molecular chaperones that bind and release peptides in an ATP dependent manner. Hsp70s participate in a diverse set of cellular processes such as protein translocation into organelles, protein folding, rearranging multiprotein complexes, disaggregation and targeting misfolded substrates to the UPP. In spite of the diverse functions of the Hsp70s, they all share a similar structural organization. Hsp70s can be divided into three regions: an N-terminal ATPase domain (NBD), a peptide binding domain (SBD), and a C-terminal lid (Flaherty et al., 1990; Zhu et al., 1996). The structural information obtained using a bacterial Hsp70 (DnaK) and a yeast Hsp70 (Ssa1p) indicate that hydrophobic peptides are bound in a channel formed by the peptide binding domain. This channel is gated by a flexible alpha-helical lid whose conformation is determined by the ATP binding status of the ATPase domain (Figure 8). The Hsp70s utilize the



energy from ATP hydrolysis to change the position of the lid domain into a conformation that favors high substrate affinity. However, Hsp70s are inherently weak ATPases and their activity can be stimulated by another class of molecular chaperones, the Hsp40s (see below, section 1.3.1.2) (Ha and McKay, 1994; McCarty et al., 1995; Theyssen et al., 1996). Hsp70s have been shown to preferentially bind hydrophobic polypeptides (S.Blond-Elguindi, 1993, GC Flynn, 1991, S. Rudiger, 2001) The yeast genome encodes for 12 different Hsp70s with 3 main cellular locations: the cytoplasm (*SSA1-4*, *SSB1,2*, *SSZ1*), mitochondria (*SSQ1*, *SSC1*, *ECM10*) and the ER (*LHS1* and *KAR2*). In this section I will focus on two cytoplasmic Hsp70 families, the *SSA* and *SSB* proteins.

Stress Seventy subfamily A (*SSA*) proteins are yeast cytosolic Hsp70s that bind newly formed polypeptides to assist with folding and prevent aggregation, and target aberrantly folded polypeptides to the proteasome for degradation (Cyr, 1995; Langer et al., 1992; Ngosuwan et al., 2003). This family of Hsp70s is also involved in post-translational translocation of polypeptides into the ER (Chirico et al., 1998; Deshaies et al., 1988; Ngosuwan et al., 2003). *Ssa1p* and *Ssa2p* are constitutively expressed while *Ssa3p* and *Ssa4p* are stress inducible. The *SSA* family of genes is essential however a high level of expression of one of the proteins is enough to maintain viability. Because the *Ssa* proteins are functionally redundant, many studies have been conducted in a strain that is deleted for the genes encoding all four *Ssa* proteins, but is covered by a plasmid containing the temperature sensitive *ssa1-45* allele (Becker et al., 1996).

Hsp40s have been shown to assist with the protein folding and disaggregation activity of *SSA1*. The most studied *Ssa* protein-Hsp40 interaction related to protein folding and degradation



### **Figure 8: The Structure Of Hsp70 And The Hsp70 ATPase Cycle**

A: The ATPase or Nucleotide Binding Domain (NBD) of bovine Hsc70 is comprised of two large globular subdomains (I and II) that can be divided into four subdomains (IA, IB, IIA, IIB). The subdomains are arranged around a cleft that contains the nucleotide binding site and interacts with the  $Mg^{2+}$  and two  $K^{+}$  ions in the structure (Figure from (Flaherty et al., 1990; Mayer and Bukau, 2005)).

B: The substrate binding domain (SBD) of DnaK in complex with substrate contains an alpha helical lid which encloses the substrate peptide in the hydrophobic binding pocket. It is proposed that ATP binding creates a conformational change in the lid that allows for substrate release from the binding pocket (Figure from (Mayer and Bukau, 2005; Zhu et al., 1996)).

C: The Hsp70 ATPase cycle: The alpha helical lid of ATP-bound Hsp70 is in an open conformation that supports low substrate affinity for the chaperone. The substrate protein is brought to Hsp70 by Hsp40 and the J domain of the Hsp40 stimulates the ATPase activity of the NBD. ATP hydrolysis by Hsp70 initiates a conformational change in the lid domain of Hsp70 that favors tight substrate binding by the chaperone. A nucleotide exchange factor (NEF) stimulates the release of ADP from the Hsp70, thus causing a conformational change in the lid domain to the low substrate affinity state. The nucleotide free Hsp70 is able to bind ATP and begin the refolding cycle again.

is Ydj1p (also see below, section 1.3.1.2) (Becker et al., 1996; Ngosuwat et al., 2003; Taxis et al., 2003). Ssa1p and Ydj1p are reported to function in a complex with Hsp104 and the small heat shock proteins, Hsp26 and Hsp42, to disaggregate proteins (Cashikar et al., 2002; Glover and Lindquist, 1998). However, another Hsp40, Hlj1p is thought to have redundant functions with Ydj1p for modulating Ssa1p activity (Youker et al., 2004).

Yeast cells containing the temperature sensitive *ssa1-45* allele are able to support the degradation of soluble ERAD substrates, such as CPY\*, that require retrotranslocation from the ER at the nonpermissive temperature (Brodsky et al., 1999). However, the cytosol from *ssa1-45* cells is unable to refold luciferase in vitro, suggesting a function for Ssa1p in the folding status of cytosolic soluble proteins. Subsequent experiments have shown that Ssa1p is necessary for the proteasomal degradation of a translocation deficient CPY\* protein and the cytosolic von Hippel-Lindau tumor suppressor (VHL) (McClellan et al., 2005; Melville et al., 2003; Park et al., 2007).

While Ssa1p is a cytosolically localized Hsp70, equal amounts of the chaperone are found free in the cytosol and associated with ER derived microsomes (Brodsky et al., 1999). Subsequently, a link between ApoB and the ERAD of polytopic membrane proteins with large cytoplasmic loops was established. For example, Hsp70 contributes to CFTR ERAD in yeast and mammals (Jiang et al., 1998; Zhang et al., 2001). In addition, mammalian Hsp70 suppresses the aggregation of the nucleotide binding domain 1 of CFTR in vitro (Meacham et al., 1999; Strickland et al., 1997). Two other polytopic membrane proteins that require Ssa1p for degradation are PMA1p-D738S and Ste6p\* (Han et al., 2007; Hoyer et al., 2004; Vashist and Ng, 2004). Furthermore, CFTR, PMA1p-D738S and Ste6p\* also had reduced polyubiquitination in *ssa1-45* mutant cells, suggesting that Ssa1p is required for the recognition of the substrate by the ubiquitination machinery (Han et al., 2007; Nakatsukasa et al., 2008). Also, ApoB requires

Hsp70 for polyubiquitination in mammalian cells and degradation in both yeast and mammalian systems (Fisher et al., 1997; Gusarova et al., 2001; Zhou et al., 1995). However, the contributions of the factors with which Hsp70 interacts, such as Hsp40s, small heat shock proteins, and nucleotide exchange factors during ApoB degradation is currently not known. This represents one topic of this dissertation.

In addition to the Ssa family of Hsp70s, there is another class of cytosolic Hsp70s, the Ssb proteins, that are pertinent to the research reported in this dissertation. There are 2 Ssb Hsp70s in the yeast genome, *SSB1* and *SSB2*, and they share 99% sequence identity; therefore it is not surprising that they are functionally redundant (Boorstein et al., 1994; Nelson et al., 1992). Yeast containing deletions of both *SSB1* and *SSB2* are viable but do exhibit a slight growth defect and are sensitive to translation-inhibiting drugs (Nelson et al., 1992). The Ssb chaperones are reported to function in a ribosome associated complex with Zuo1p (an Hsp40) and Ssz1p (another Hsp70) (Huang et al., 2005; Hundley et al., 2002). Ssb1p was found to interact with the ribosome near the exit site and to nascent polypeptide chains by cross linking studies. In addition, Ssb1p has been found to associate with both translating and nontranslating ribosomes, suggesting that it impacts the earliest point during the translation of a protein (Pfund et al., 1998). The Ssz1p/Zuo1p interaction is required for the interaction of Ssb1p with the nascent polypeptide chains (Gautschi et al., 2001; Gautschi et al., 2002). In spite of the interaction with the exit site of the ribosome and the link to the early stages of protein biogenesis, no role for Ssb1p and Ssb2p in ERAD has been determined.

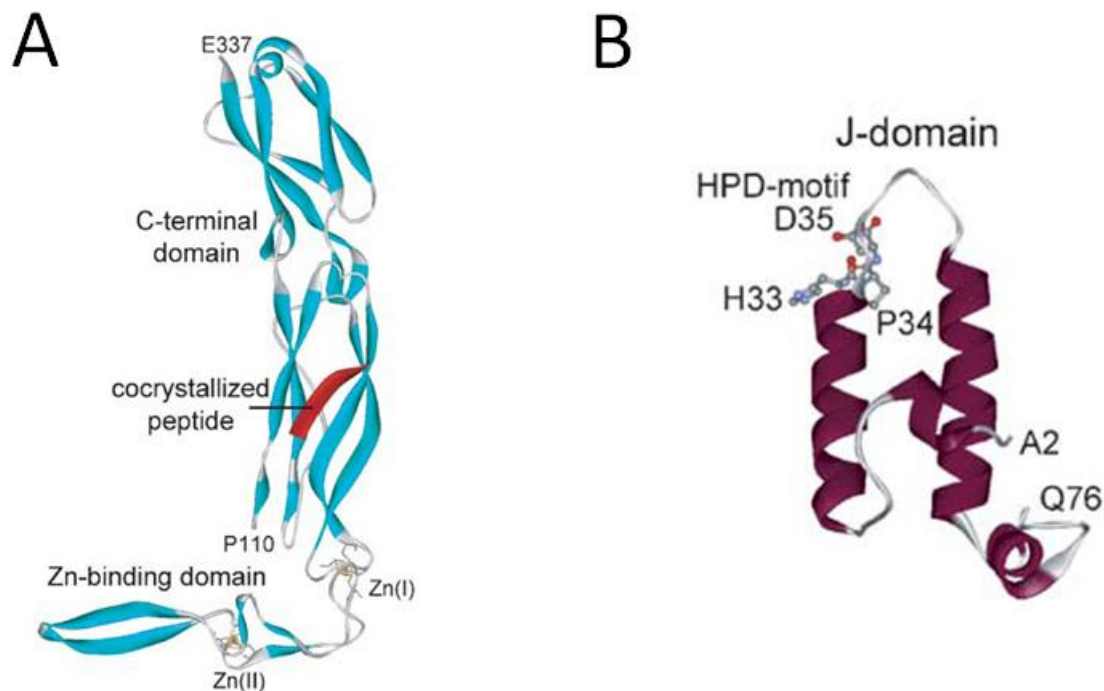
#### **1.3.1.2 Hsp40**

The Hsp40 molecular chaperone family all share a ~70 amino acid sequence called the J domain that mediates interactions with and is critical for stimulating the ATPase cycle of Hsp70s (Figure

8) (McCarty et al., 1995). In addition, data suggest that some Hsp40s can bind peptides directly and are involved in substrate transfer to Hsp70 (Figure 9) (Rudiger et al., 2001). It has been shown that some Hsp40s require both their J domain and peptide binding domain to assist Hsp70 with protein folding in vitro (Levy et al., 1995; Lu and Cyr, 1998). Structural studies of the J domain of bacterial DnaJ, human Hdj1p, and the SV40 large T antigen reveal that the J domain is composed of 4  $\alpha$ -helices, two of which are packed tightly together to form a finger like projection. The histidine-proline-aspartic acid (HPD) tripeptide motif that is critical for J domain-Hsp70 interaction is located on the tip of this finger (Figure 9) (Berjanskii et al., 2000; Hill et al., 1995; Pellicchia et al., 1996; Qian et al., 1996; Szyperski et al., 1994; Tsai and Douglas, 1996).

The Hsp40s are classified into three groups based on structural features besides the J domain: type I are the most similar to DnaJ containing a J domain, a glycine/phenylalanine-rich region that assists with the Hsp40-Hsp70 interaction, and a cysteine-rich peptide binding pocket; type II lack the cysteine-rich region, but contain both the J domain and the glycine/phenylalanine-rich region; type III lack both the cysteine-rich region and the glycine/phenylalanine-rich region (Cheetham and Caplan, 1998).

Two cytosolic Hsp40s that regulate the ATPase activity of Ssa1p and subsequently the Hsp70s role in translocation and ERAD are Ydj1p and Hlj1p (Cyr, 1995; Cyr and Douglas, 1994; Cyr et al., 1992; Huyer et al., 2004; Lu and Cyr, 1998; Tsai and Douglas, 1996; Youker et al., 2004). Ydj1p is a type I Hsp40 that is linked to the ER membrane by prenylation. Deletion of Ydj1p caused a severe growth defect in yeast, therefore in my studies I utilized a temperature sensitive allele, *ydj1-151*, that has been shown to reduce both translocation and



**Figure 9: The Structure Of An C-Terminal Hsp40 Fragment and The J domain**

A: A crystal structure of a truncated fragment of the yeast Hsp40, Ydj1p, lacking the N-terminal J domain. The protein is shown in complex with a peptide (shown in red) (Figure from (Li et al., 2003; Mayer and Bukau, 2005)).

B: A NMR structure of the J domain of DnaJ, the bacterial Hsp40. The invariant HPD motif at the tip of the J domain is highlighted. This motif is essential for the interaction of the J domain with Hsp70 and subsequent stimulation of Hsp70 ATPase activity (Figure from (Mayer and Bukau, 2005; Pellicchia et al., 1996)).

ERAD (Becker et al., 1996; Brodsky et al., 1999; Caplan et al., 1992; Taxis et al., 2003; Youker et al., 2004). In contrast, Hlj1p is a type II Hsp40 anchored to the ER membrane by a C-terminal tail and cells lacking this Hsp40 are viable (Beilharz et al., 2003; Huyer et al., 2004). The function of Hlj1p and Ydj1p in ERAD are thought to be redundant because single deletions had no effect on the degradation of CFTR and Ste6p\*, however the proteins were stabilized in the *ydj1-151hlj1Δ* strain (Huyer et al., 2004; Youker et al., 2004). Like Ssa1p, the function of Ydj1p and Hlj1p appear to be dispensable for ER luminal soluble ERAD substrates such as CPY\* (Huyer et al., 2004). In addition, like their functional partner, Ssa1p, they appear to be required for the polyubiquitination of polytopic membrane proteins (Nakatsukasa et al., 2008). It is currently unknown if the cytosolic Hsp40s interact with or impact the degradation of ApoB.

### **1.3.1.3 Hsp70 Nucleotide Exchange Factors**

Another class of proteins that contributes to the regulation of the ATPase cycle of Hsp70, and thus regulates the peptide binding activity of the chaperone, are the nucleotide exchange factors (NEFs) (Figure 8). Ssa1p has three NEFs that have been identified: Snl1p, Fes1p, and Sse1p. Sse1p is an Hsp110 that has additional functions beyond its activity as a NEF and will be discussed in detail below in section 1.3.1.5.

The first NEF identified in yeast for the cytosolic Hsp70, Ssa1p, was Fes1p, a protein with similarity to the ER luminal NEF for Kar2p (Kabani et al., 2002). Fes1p preferentially associates with ADP bound Ssa1p and inhibits the Ydj1p-mediated activation of the ATPase activity of Ssa1p. *fes1Δ* cells exhibited phenotypes such as cycloheximide sensitivity and a general translation defect, suggesting a role for the NEF in protein translation. However, a role for Fes1p in Ssa1p-mediated ERAD or translocation was not reported (Kabani et al., 2002).



Another yeast Hsp70 NEF, Snl1p, was found in a search for yeast proteins with sequence similarities to the mammalian Hsp70 NEF, Bag-1 (Sondermann et al., 2002). Bag-1 had been shown to inhibit Hsp70 refolding activity and promote ADP dissociation in vitro (Gassler et al., 2001). Snl1p bound two different cytosolic Hsp70s, Ssa1p and Ssb1p (Sondermann et al., 2002). Snl1p also participates in nuclear pore function, a characteristic that is not shared by Sse1p or Fes1p (Ho et al., 1998). However, there is no reported contribution of Snl1p to ERAD (J. Bennett and J Brodsky, unpublished data). Recently, a screen for mutants that block [URE3] prion propagation isolated both Sse1p and Fes1p, but not Snl1p. In addition, only Sse1p interfered with [PSI<sup>+</sup>] prion propagation (Kryndushkin and Wickner, 2007). Overall, current data suggest that there are functional differences between the NEFs, and the nature of these differences still need to be defined.

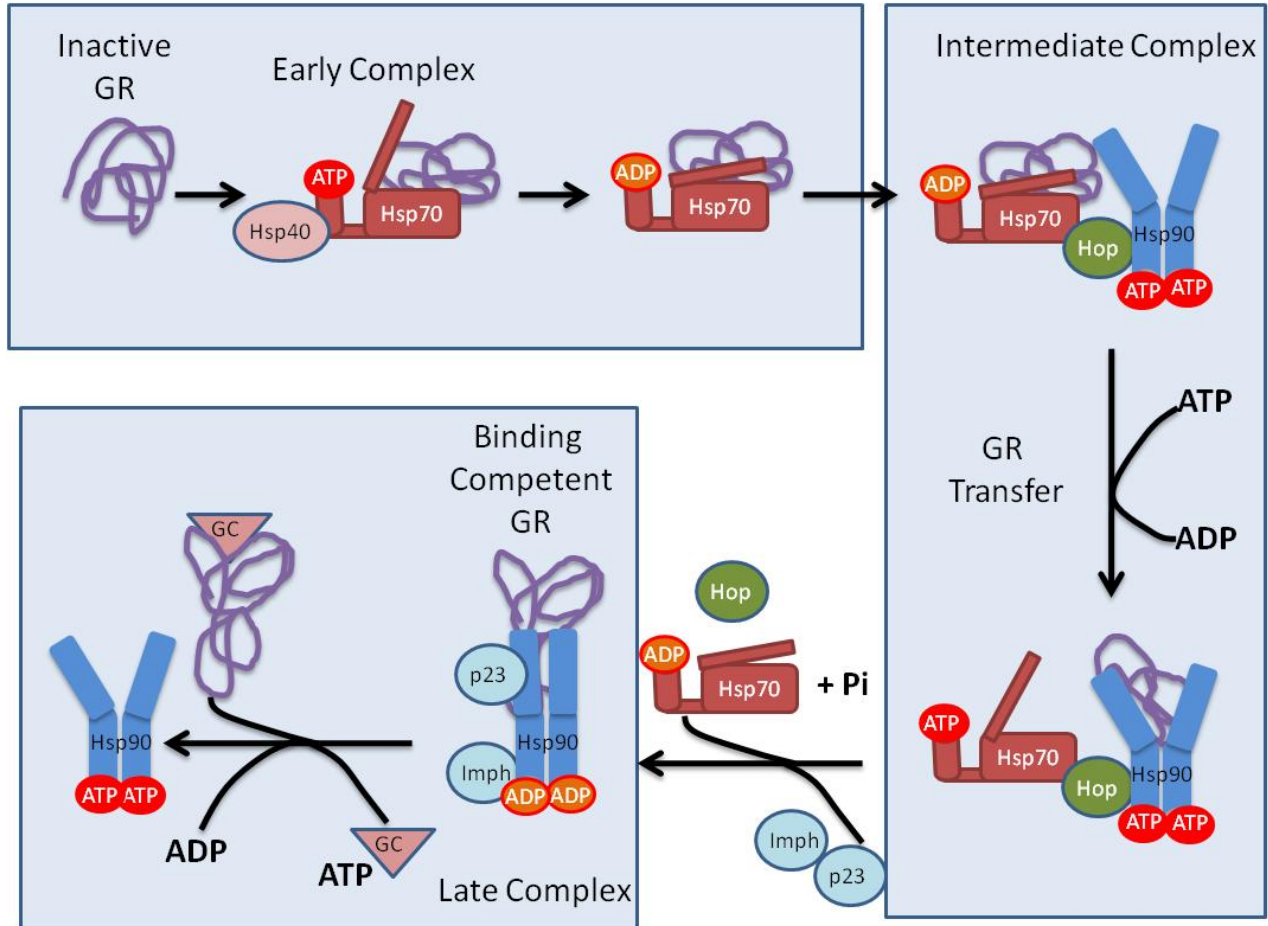
#### **1.3.1.4 Hsp90**

Hsp90 is one of the most abundant proteins in the cell, comprising 1-2% of the total protein of unstressed cells (Lai et al., 1984; Welch and Feramisco, 1982). This chaperone helps fold a diverse set of client proteins, such as transcription factors, steroid hormone receptors, and protein tyrosine kinases. For example, Hsp90 has been found to be essential for the proper folding of the glucocorticoid receptor and vSrc in both mammalian and yeast systems (Goeckeler et al., 2002; Nathan and Lindquist, 1995; Smith et al., 1990a; Smith et al., 1990b; Smith et al., 1992; Smith and Toft, 1993). Hsp90 does not act alone in regulating the folding of these client proteins, but rather functions in a series of complexes with other chaperones and co-chaperones to achieve the proper folding of the polypeptide. The glucocorticoid receptor (GR) folding pathway is well characterized (Figure 10). There are 3 chaperone complexes that associate with the protein during its folding and activation in mammalian cells, although there are comparable complexes

in yeast. The “early complex” consists of GR bound by Hsp70 and Hsp40. In the “intermediate complex”, a co-chaperone, Hop (Sti1p in yeast) bridges an interaction between Hsp70 and Hsp90 and assists with substrate transfer from Hsp70 to Hsp90. Subsequently, Hop and Hsp70 are released from the complex in exchange for the co-chaperone, p23 (Sba1p in yeast) and an immunophilin and Hsp90 hydrolyzes ATP to ADP. These changes all function to stabilize the Hsp90-GR interaction. The GR is now in a hormone binding competent state and upon hormone interaction the GR will be released from the chaperone complex (Dittmar et al., 1997; Kosano et al., 1998; Smith and Toft, 1993; Smith et al., 1995a; Wegele et al., 2004).

Hsp90 is composed of 3 major domains: an N-terminal ATPase, a middle domain which functions as the substrate binding pocket, and a C-terminal dimerization domain (Wegele et al., 2003). Hsp90 is a dimer in its peptide binding-competent state and dimerization of the chaperone is regulated by the ATPase domain (Chadli et al., 2000; Maruya et al., 1999; Prodromou et al., 1997; Richter and Buchner, 2001). Geldanamycin and Radicicol are two compounds that inhibit Hsp90 folding activity by interacting with the ATP binding pocket and thus blocking the essential ATPase activity of the chaperone (Roe et al., 1999). The C-terminal dimerization domain is not only important for mediating contacts between the two Hsp90 subunits, but also contains a MEEVD motif that functions as a binding site for tetratricopeptide (TPR) repeat containing co-chaperones that regulate Hsp90 activity, such as Sti1p (Chen et al., 1996; Chen et al., 1998; Owens-Grillo et al., 1996; Young et al., 1998).

In spite of the link connecting Hsp90 function to a mammalian E3 ligase, known as CHIP, the known role for Hsp90 in ERAD is limited to a few substrates (Table 2) (Connell et al., 2001). CFTR has been shown in mammalian and yeast cells to be stabilized by Hsp90 and requires the chaperone for maturation (Loo et al., 1998; Youker et al., 2004; Zhang et al., 2002).



### **Figure 10: Glucocorticoid Receptor Folding By The Hsp90 Complex**

Nascent unfolded glucocorticoid receptor (GR) is in an inactive form, unable to recognize and bind the cognate hormone, glucocorticoid (GC). The misfolded GR is bound by an early complex containing Hsp40 and Hsp70. Hsp40 J domain stimulated ATP hydrolysis by Hsp70 initiates tight binding of GR to the chaperone. Subsequently, the co-chaperone Hop bridges an interaction between Hsp70 and Hsp90 forming the intermediate complex. Hop then mediates substrate transfer from Hsp70 to Hsp90. The co-chaperones immunophilin (Imph) and p23 interact with Hsp90, dislodging Hsp70 and Hop from the intermediate complex. Hsp90 ATP hydrolysis occurs, creating a tight interaction with the GR substrate protein. Imph, p23, and Hsp90-ADP form the late complex. If the GR is folded properly, it will be able to interact with GC and the GR will change conformation, stimulating release of the GR-GC complex. Hsp90 is then free to bind another substrate polypeptide.

Interestingly, Hsp90 had differential effects on protein degradation as Hsp90 functions as a pro-stabilizing factor for firefly luciferase and a pro-degradation factor for mutant insulin receptors (Imamura et al., 1998; Schneider et al., 1996). The contribution of Hsp90 co-chaperones to CFTR stability is still an open area of investigation, however the data from a yeast system indicate that Sti1p (Hop) and Sba1p (p23) do not regulate the degradation of CFTR (Youker et al., 2004). Another Hsp90 co-chaperone, Aha1p, that accelerates the ATPase activity of Hsp90 was found to preferentially interact with the misfolded protein  $\Delta$ F508 CFTR in mammalian cells (Lotz et al., 2003; Panaretou et al., 2002; Wang et al., 2006). Reducing the intracellular levels of Aha1p, without changing the levels of other Hsp90 co-chaperones, resulted in an increase in the stability and trafficking of CFTR (Wang et al., 2006). These data suggest that Hsp90 co-chaperones have a role in regulating the maturation and degradation of Hsp90 client proteins. Interestingly, like Hsp70, Hsp90 is important for targeting ApoB for proteasomal degradation. However unlike Hsp70, the activity of Hsp90 appears to function upstream of polyubiquitination because altering Hsp90 levels did not alter the ubiquitination status of ApoB (Gusarova et al., 2001). The role of Hsp90 co-chaperones in regulating the maturation and ERAD of ApoB is one topic of this dissertation.

#### **1.3.1.5 Hsp110**

Hsp110 is a conserved cytoplasmic molecular chaperone that has been identified in a variety of organisms (Chung et al., 1998; Lee-Yoon et al., 1995; Oh et al., 1997; Plesofsky-Vig and Brambl, 1998; Storozhenko et al., 1996; Yasuda et al., 1995). Hsp110s have been shown to be NEFs for Hsp70s, interact functionally with chaperone complexes such as the Hsp90 complex and function as ‘holdases’, chaperones that hold proteins in a folding competent state but are

unable to refold polypeptides by itself (Brodsky et al., 1999; Dragovic et al., 2006b; Goeckeler et al., 2002; Liu et al., 1999; Oh et al., 1999; Raviol et al., 2006a; Raviol et al., 2006c; Shaner et al., 2006).

The cytosolic yeast Hsp110, Sse1p, was originally identified as a calmodulin binding protein and pulled out in a screen for multicopy suppressors of a heat shock sensitive phenotype (Mukai et al., 1993; Shirayama et al., 1993). There are two Sse proteins in yeast. Sse1p is constitutively expressed but higher levels of expression can be induced by heat stress. The endogenous expression of Sse2p is very low, but its synthesis is greatly increased in heat stressed cells (Mukai et al., 1993; Shirayama et al., 1993). This corresponds with data in which overexpression of mammalian Hsp110 in CHO cells increased the thermotolerance of the mammalian cell line (Oh et al., 1997). The phenotypes of the deletion mutants vary in that the *sse1Δ* cells have a slow growth phenotype, but the *sse2Δ* cells exhibit no phenotype. This is probably due to their relative expression levels as the *sse1Δsse2Δ* cells show an equivalent amount of growth defect and heat sensitivity as *sse1Δ* cells (Mukai et al., 1993). Sse1p shares 24% overall identity with a mammalian Hsp110 (Easton et al., 2000). Interestingly, the expression of mammalian Hsp110 was unable to rescue the slow growth defect of *sse1Δ* cells, suggesting that there are functional differences between mammalian and yeast Hsp110s (Liu et al., 1999). However, overexpression of mammalian Hsp110 can suppress the toxicity of an aggregation prone poly-glutamine protein in Cos-7 cells and purified Sse1p can stimulate nucleotide exchange in mammalian Hsp70 in vitro, indicating that the functions of Hsp110 in yeast and mammals are not completely divergent (Brodsky et al., 1999; Ishihara et al., 2003; Liu et al., 1999; Oh et al., 1997; Shaner et al., 2006).

Hsp110 has been linked to the Hsp90 complex by both biochemical and genetic data. Cells lacking both Sse1p and Stilp (Hop) exhibit a slower growth phenotype than cells containing the single deletions (Liu et al., 1999). In addition, *sse1Δ* cells are sensitive to Hsp90 inhibitors geldanamycin and macbesin. In immunoprecipitation experiments, Sse1p was identified with Hsp90 complexes by mass spectrometry. The Hsp90 complex client proteins, glucocorticoid receptor, human androgen receptor and v-Src kinase, require Sse1p for their maturation in yeast and VHL, a substrate targeted to the UPP via the Hsp90 complex, requires Sse1p for degradation (Goeckeler et al., 2002; Liu et al., 1999; McClellan et al., 2005). However, CFTR, a protein that requires Hsp90 for ERAD, does not require Sse1p or other Hsp90 co-chaperones for degradation (Youker et al., 2004). The Sse1p-Hsp90 complexes isolated contain the Hsp70, Ssa1p, and an Hsp40, Ydj1p (Liu et al., 1999). Subsequently, Sse1p was isolated as a high copy suppressor of a temperature sensitive allele of the Hsp40 associated with the Hsp90 complex, Ydj1p, and an *sse1Δydj1Δ* strain is inviable (Goeckeler et al., 2002; Shaner et al., 2006). These data suggest that while Hsp110 is important for the degradation of some Hsp90 client proteins it may also cooperate with Hsp40 to regulate the activity of Hsp70.

As a result, the focus on Hsp110 function has shifted from its association with the Hsp90 complex to the interaction with and regulation of cytosolic Hsp70s. Sse1p is reported to be in heterodimeric complexes with the cytosolic Hsp70s, Ssa1p and Ssb1p (Shaner et al., 2005; Yam et al., 2005). In addition, mammalian Hsp110 has been isolated in complexes with Hsp70 (Ishihara et al., 1999; Wang et al., 2000). In fact, it has been proposed that due to the ratio of Sse1p to Ssa1p (1:9) and to Ssb1p (1:4), all of the Sse1p in the cell is in a complex with either Hsp70 and is not likely found in an unbound form (Ghaemmamghami et al., 2003; Shaner et al., 2005; Yam et al., 2005). In any event, the Ssa/Sse and Ssb/Sse complexes appear to interact

with polypeptides at different stages of their biogenesis. Because Ssb1p is the Hsp70 in the ribosome associated complex it is not surprising that the Ssb/Sse complex is found bound co-translationally to polypeptides. While the Ssa/Sse complex interacts primarily post-translationally with polypeptides, it can also compensate for a loss of Ssb1p expression by binding polypeptides co-translationally. Nevertheless, Sse1p is thought to regulate the peptide binding activity of both Ssa1p and Ssb1p because in the *sse1Δ* cells more of the Hsp70s are found bound to polypeptides (Yam et al., 2005). The interaction of Hsp110 with Hsp70 impacts the function of Hsp70 because Hsp110 can stimulate the Hsp70-Hsp40 refolding of heat denatured luciferase and the ATPase activity of Hsp70 in the presence of Hsp40 in vitro (Brodsky et al., 1999; Dragovic et al., 2006b; Oh et al., 1997; Shaner et al., 2005; Yam et al., 2005).

As previously mentioned there are three Hsp70 NEFs in yeast, Sse1p, Snl1p and Fes1p. Only Fes1p has been examined as being functionally redundant with Sse1p as a NEF. Indeed, overexpression of Fes1p, can partially rescue the slow growth phenotype of *sse1Δsse2Δ* cells, but the cells are still sensitive to stress conditions (Raviol et al., 2006c). In addition, while *sse1Δyjd1Δ* cells are inviable, *fes1Δyjd1Δ* cells are able to survive (Shaner et al., 2006). Overall, these genetic data suggest that Sse1p has additional essential cellular functions that Fes1p cannot restore. Another functional difference between Sse1p and Fes1p is that Sse1p preferentially binds Ssa1p-ADP and the binding cannot be increased by the addition of ATP. In contrast, Fes1p preferentially binds Ssa1p-ATP (Kabani et al., 2002; Raviol et al., 2006a). Finally, Fes1p has no significant effect on the rate of firefly luciferase refolding by Hsp70 and Hsp40 in vitro and is not found in Hsp90/Hsp70 substrate complexes (Dragovic et al., 2006b; Liu et al., 1999). These collective data suggest that the function of Sse1p is unique compared to other Hsp70

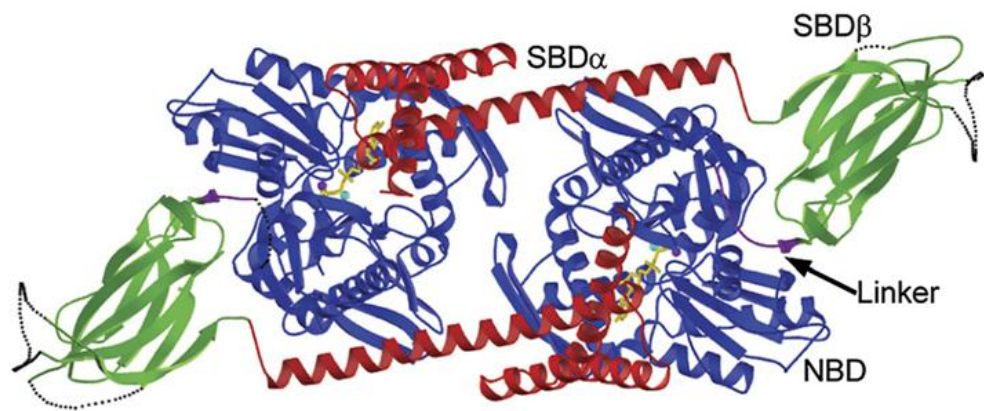


NEFs and that these functions may be due to the holdase activity of the chaperone and its interactions with the Hsp90 complex.

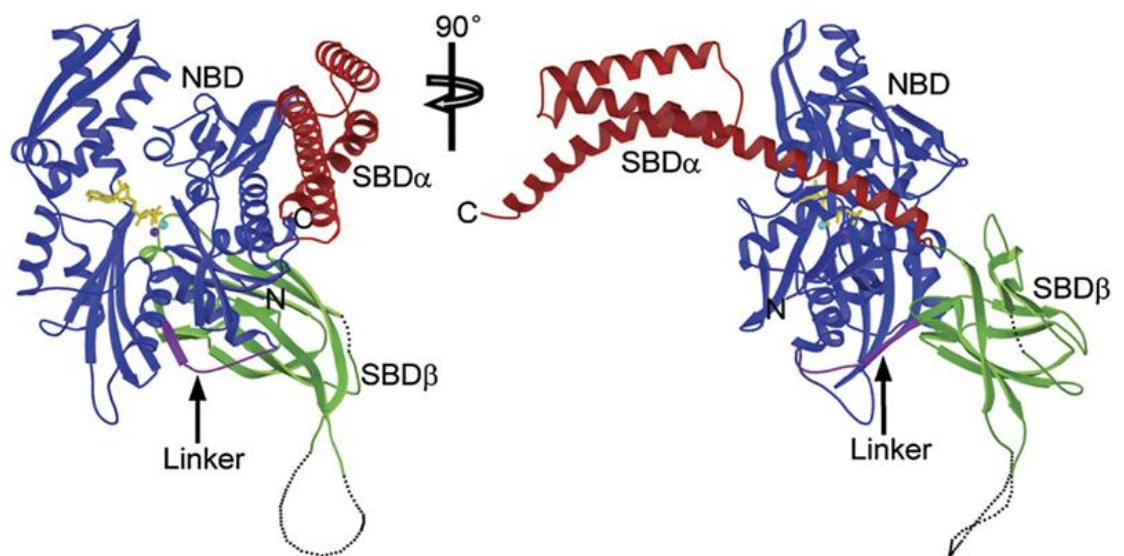
The Hsp110 proteins have structural similarities to the Hsp70 family of molecular chaperones with an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD); however, the larger size of the proteins is due to a loop inserted between the NBD and SBD and a C-terminal extension. The NBD shows sequence similarity (~30%) to Hsp70, while the SBD does not show great amino acid similarity, but is predicted to have a similar secondary sequence. Recently, the crystal structure of a homodimer of Sse1p bound to ATP was solved (Liu and Hendrickson, 2007) (Figure 11). The amino acid sequence of the protein was intact except for a 34 amino acid truncation at the C-terminus. Previously, a C-terminal 44 amino acid truncation mutant of Sse1p was shown to be fully functional (Shaner et al., 2004). Therefore, the Sse1p structural information may be more physiologically relevant than the solved structures of Hsp70s, which required mutating several residues in order to stabilize the structure and were solved either in the absence of nucleotide or in the ADP bound form (Revington et al., 2005; Swain et al., 2007; Vogel et al., 2006a; Vogel et al., 2006b).

The structure of Sse1p was solved in its ATP bound form probably because interaction with nucleotide stabilizes SBD-NBD interactions (Raviol et al., 2006c; Shaner et al., 2006). The SBD and NBD are intimately associated in the ATP bound state, corresponding to data obtained that the SBD and NBD can functionally interact in yeast when expressed *in trans* (Liu and Hendrickson, 2007; Shaner et al., 2004). The association between the NBD and SBD is mediated by 16 hydrogen bonds that maintain the orientation of 2 critical residues, Lys 69 and Asp 174 (Figure 11). These residues in the Hsp70 NBD are the key catalytic residues for ATP

A



B



**Figure 11: Crystal Structure Of Hsp110 In The ATP Bound State**

A: Crystal structure of the homodimer of the yeast Hsp110, Sse1p. The interaction between the two Sse1p proteins is mediated by tip-to-tip contacts of the nucleotide binding domains (NBD) (Structure from (Liu and Hendrickson, 2007)).

B: Extrapolated crystal structure of an Sse1p monomer. The structure of Hsp110 shows similarities to Hsp70 with an alpha helical lid, an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD). However, Hsp110 is a larger size than Hsp70 due to an extended linker domain and a C-terminal extension. Because the crystal structure was solved in the ATP bound state, the alpha helical lid has obtained an open conformation in relation to the SBD. The interactions between the NBD and SBD are conserved between Hsp70 and Hsp110 (Structure from (Liu and Hendrickson, 2007)).

hydrolysis. Interestingly, the hydrogen bonding in the Sse1p structure causes these residues to be displaced from their proposed localization in the Hsp70 NBD (Liu and Hendrickson, 2007). This may account for the slower ATP hydrolysis rate of Sse1p compared to Ssa1p (Raviol et al., 2006c).

Previous structural and biochemical data have suggested that the conformation of the lid domain of the SBD of Hsp70 is regulated by the nucleotide binding status of the NBD (Flaherty et al., 1990; Zhu et al., 1996). The residues that become buried by the SBD-NBD interactions in the ATP bound state are highly conserved between Hsp70s and Hsp110s suggesting they may have critical function in the communication between the domains of the proteins (Liu and Hendrickson, 2007). Interaction data from the Hsp110 structure was utilized to generate Hsp70 and Hsp110 constructs with individual mutations at the conserved residues buried by the SBD-NBD contacts. The mutants were then examined for thermotolerance to determine if the chaperone activity of the proteins was compromised by the mutations. The viability of the Hsp70 mutants during heat stress was impaired in 8 of 9 mutants examined, suggesting that these residues are important for the regulation of SBD activity by the NBD. However, the Hsp110 mutant showed subdomain specificity in regard to importance of the residues buried by the NBD-SBD interaction. Hsp110 proteins with mutations of the contacts between the SBD $\alpha$  subdomain and the NBD showed heat stress sensitivity phenotypes. However, when residues in the NBD-SBD $\beta$  contact sites were mutated, there was no effect on the thermotolerance of the yeast expressing the mutant protein. These data suggest that there are differences in the importance of the buried residues in the NBD-SBD interface between Hsp70s and Hsp110.

Sse1p can homodimerize and the crystal structure reveals that the dimerization is mediated by tip to tip contacts of the NBDs (Liu and Hendrickson, 2007). This correlates well

with data that indicates that while the NBD of Sse1p does not appear to be essential for its function as an in vitro holdase, the activity of the domain is important for complex formation with Hsp70 and is required to rescue the slow growth phenotype of *sse1Δ* and *ydj1-151* cells (Oh et al., 1999; Shaner et al., 2006; Shaner et al., 2004; Shaner et al., 2005). When residues in the dimerization domains were mutated, the resultant Hsp110s exhibited no growth defect during heat stress, suggesting that this interaction is not critical for Sse1p function. Although the dimerization status of the mutants was not examined, it should be noted that Sse1p is proposed to function as an Hsp70/Hsp110 heterodimer in the cell and the exact contact sites of this interaction are not known (Shaner et al., 2005; Yam et al., 2005).

### **1.3.2 Structure and Function of Select ER Luminal Molecular Chaperones**

Five classes of ER luminal molecular chaperones and their co-factors will be discussed in this section. For a complete list of the chaperones and co-factors covered see Table 1. The ERAD substrates and the chaperones that regulate their degradation that are discussed in the following sections are listed in Table 2.

#### **1.3.2.1 Hsp70**

Like the cytosolic Hsp70s discussed above, the ER luminal Hsp70, Kar2p (BiP in mammalian cells), regulates many cellular processes such as translocation, retrotranslocation, retaining the solubility of misfolded polypeptides, and targeting aberrantly folded proteins to ERAD (Alder et al., 2005; Denic et al., 2006; Kabani et al., 2003; Nishikawa et al., 2001; Taxis et al., 2003). Due to the breadth of functions in which Kar2p functions, it is not surprising that the gene encoding this Hsp70 is essential for viability (Normington et al., 1989; Rose et al., 1989). Many of the

cellular processes in which Kar2p participates in have been examined using temperature sensitive or truncation mutants. In my studies, I utilized a temperature sensitive mutant of Kar2p, *kar2-I*, that has previously been shown to have impaired function in ERAD (Brodsky et al., 1999; Hoyer et al., 2004; Kabani et al., 2003).

The overall structure of the ER luminal Hsp70s are proposed to be similar to the cytosolic Hsp70s with three distinct domains (see above). In yeast, the cellular function of Kar2p depends upon its interaction with its three cognate J domain containing proteins: the membrane localized Hsp40s Sec63p and Jem1p and the soluble Hsp40, Scj1p (see below). The interaction between Kar2p and Sec63p is essential for the role of the Hsp70 in translocation, while Scj1p and Jem1p both are involved in ERAD (Nishikawa et al., 2001). In mammals, BiP is proposed to be the only protein necessary and sufficient to gate to the translocon pore, an essential function for maintaining the oxidizing environment of the ER (Alder et al., 2005; Hamman et al., 1998). BiP requires its substrate binding domain and its ability to hydrolyze ATP in a Sec63p stimulated manner in order to function as a gate at the translocon (Alder et al., 2005). In addition to maintaining the permeability of the pore, both BiP and Kar2p are required for both post and co-translational translocation of polypeptides into the ER lumen (Bies et al., 1999; Brodsky et al., 1995; Young et al., 2001). Like other Hsp70, BiP and Kar2p also bind hydrophobic patches on misfolded polypeptides and is essential for mediating the retrotranslocation of the soluble ERAD substrates across the ER membrane to the cytoplasm for proteasomal degradation (Blond-Elguindi et al., 1993a; Blond-Elguindi et al., 1993b; Kabani et al., 2003; Skowronek et al., 1998).

There are distinct differences between the target ERAD substrates of Kar2p compared to the cytosolic Hsp70s. Kar2p is not required for the ERAD of integral membrane proteins with

large cytosolic domains that require Ssa1p, such as CFTR, and Ste6p\* (Huyer et al., 2004; Nakatsukasa et al., 2008; Vashist and Ng, 2004; Zhang et al., 2001). However, Kar2p does function in the ERAD of the soluble, fully translocated polypeptide substrates: CPY\*, A1PiZ, and proalpha factor (Table 2) (Brodsky et al., 1999; Huyer et al., 2004; Nishikawa et al., 2001; Plemper et al., 1997). Therefore, there appears to be a distinct separation of the function of cytosolic and ER luminal Hsp70s on the ERAD of substrates based upon the location of the misfolded polypeptide. The mammalian Kar2p homolog, BiP, has also been shown to interact with ApoB during its synthesis and travel with the primordial lipoprotein to the Golgi (Zhang and Herscovitz, 2003). However, a link between the chaperone and ApoB ERAD has not been established.

#### **1.3.2.2 Hsp40**

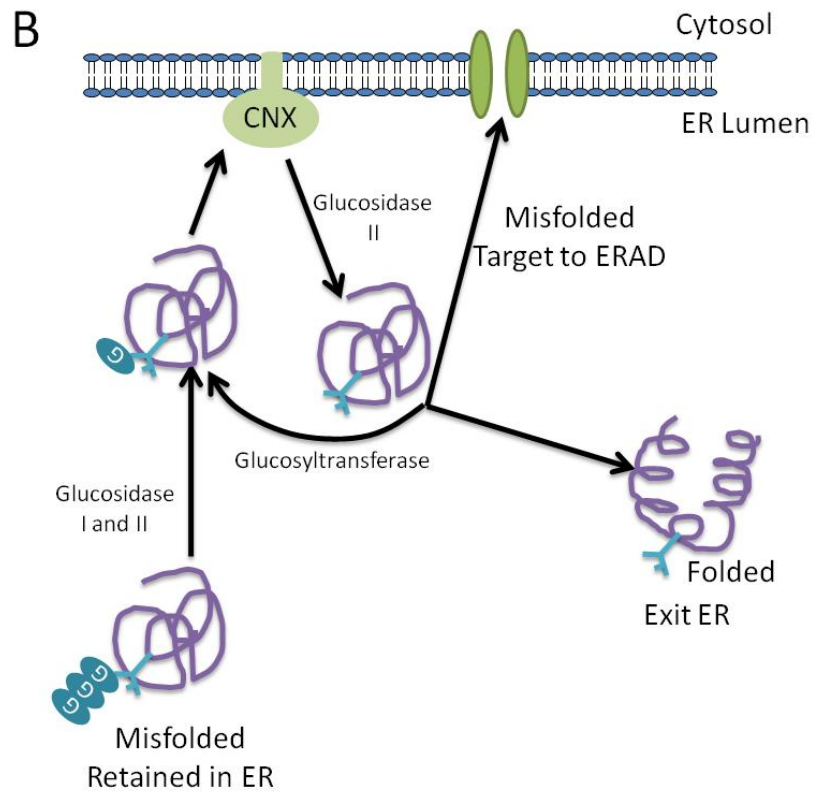
As mentioned previously, the cognate Hsp40s for Kar2p are Sec63p, Scj1p and Jem1p, and these Hsp40s are important for distinct cellular functions of Kar2p. Sec63p is a type III Hsp40 that is membrane localized and essential for viability. Sec63p assists Kar2p with translocating polypeptides into the ER lumen through the translocon, perhaps by ratcheting substrates (Corsi and Schekman, 1997; Matlack et al., 1999; Walsh et al., 2004). The soluble Hsp40, Scj1p and the membrane associated Hsp40, Jem1p, are not essential for viability, but yeast lacking both Scj1p and Jem1p are temperature sensitive, suggesting that they are functionally redundant (Nishikawa and Endo, 1997; Walsh et al., 2004). Only Scj1p and Jem1p are linked to the protein folding function of Kar2p, but not translocation, while Sec63p is only responsible for the translocation activity of the Hsp70 (Nishikawa et al., 2001; Silberstein et al., 1998; Young et al., 2001).

Yeast deleted for both Scj1p and Jem1p have a functional translocation system but the transport of substrates such as CPY to the Golgi is reduced (Silberstein et al., 1998). This suggests further that Scj1p and Jem1p are not required for the translocation activity of Kar2p. Instead they contribute to the folding activity of the Hsp70 that is necessary for secreted protein transport out of the ER. Like Kar2p, the ERAD of soluble proteins such as CPY\* and proalpha factor require the presence of both of these Hsp40s, but integral membrane proteins such as Sec61-2 require neither Scj1p nor Jem1p (Table 2) (Nishikawa et al., 2001). In addition to the difference in localization of Jem1p, a membrane associated protein, and Scj1p, a soluble protein, Jem1p is a type III Hsp40 and Scj1p is a type I Hsp40. This suggests that there may also be functional differences in the proteins. Jem1p, but not Scj1p, is reported to cooperate with Kar2p during nuclear membrane fusion during mating (Brizzio et al., 1999; Nishikawa and Endo, 1997). Therefore even though these two Hsp40s do share some cellular processes, they diverge in their structure and in some functions.

### **1.3.2.3 Calnexin and Calreticulin**

As polypeptides are translocated into the ER a core Glc<sub>3</sub>-Man<sub>9</sub>-GlcNAc<sub>2</sub> glycan is added onto the consensus sequence, Asn-Xaa-Ser/Thr, by an oligosaccharyltransferase (Figure 12). Subsequently, the core glycan is cleaved by glycosidases, releasing the two terminal glucose residues and resulting in a monoglycosylated species (Helenius and Aebi, 2001). The monoglycosylated species produced by this process is monitored in mammalian cells by two specialized ER lectin luminal molecular chaperones. In mammalian cells two of these lectins are calnexin, an integral membrane protein and calreticulin, a soluble protein. The yeast, *S. cerevisiae* express a single calnexin homolog (Parlati et al., 1995). Calnexin and calreticulin will retain polypeptides in the ER and assist with folding until the final glucose residue is cleaved and





**Figure 12: Glycan Addition To Protein Substrates And Monitoring Of Glycan Composition By Calnexin and Calreticulin**

A: The branched chain of sugars,  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ , that is added to asparagines of secreted proteins with the motif NX(S/T) (Figure from (Williams, 2006)).

B: Oligosaccharyl transferase recognizes the NX(S/T) sequence in translocating polypeptides and adds the preassembled  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  onto the asparagine residue. This core glycan is cleaved by glucosidase I and II reducing the number of glucose residues at the tip of one of the branched chains of sugars. The monoglycosylated species is recognized by the ER lectin calnexin (CNX) and bound by the molecular chaperone to facilitate the folding of the protein. Removal of the terminal glucose residue by glucosidase II results in release of the polypeptide from CNX. If the polypeptide has achieved its proper folded state it can exit the ER to continue in the secretory pathway. If the protein is still misfolded the protein can be reglycosylated with a single glucose residue by glucosyltransferase and the protein will be retained in the lectin cycle in the ER. If the misfolded protein is associated with the lectin cycle for an extended period of time it will be targeted for retrotranslocation to the cytoplasm and subsequent proteasomal degradation. For simplicity the lectin EDEM and  $\alpha$ -mannosidase are not shown in the figure.

the properly folded protein can exit the ER (Figure 12) (Hebert et al., 1995; Helenius and Aebi, 2001). If the protein is unable to fold, UDP-glucose:glycoproteintransferase (UGGT) will bind the misfolded protein and add a single glucose moiety to the glycan (Tessier et al., 2000). Prolonged time in the with lectin-glycan cycle can result in the polypeptide being targeted to ERAD for degradation (Holst et al., 1996). This occurs by the generation of a  $\text{Man}_8\text{GlcNAc}_2$  by  $\alpha$ -mannosidase (Jakob et al., 1998; Sousa et al., 1992). The lectin termed ER Degradation Enhancing  $\alpha$ -Mannose-like protein (EDEP) recognizes this particular oligosaccharide structure and targets the polypeptide for retrotranslocation to the cytosol where proteasomal degradation will occur (Molinari et al., 2003; Oda et al., 2003). As stated previously, upon entrance to the ER, ApoB is N-link glycosylated and this is important for the proper lipidation and efficient secretion of the lipoprotein particle (Harazono et al., 2005; Huang and Shelness, 1999; Liao et al., 1998; Pease et al., 1995). Although, an interaction with calnexin has been reported (Chen et al., 1998; Tatu and Helenius, 1999), the role of this cycle in the degradation of ApoB has yet to be investigated without the use of compounds that alter the glycosylation status of ER luminal polypeptides and also induce the UPR (Liao and Chan, 2001; Macri and Adeli, 1997a; Macri and Adeli, 1997b).

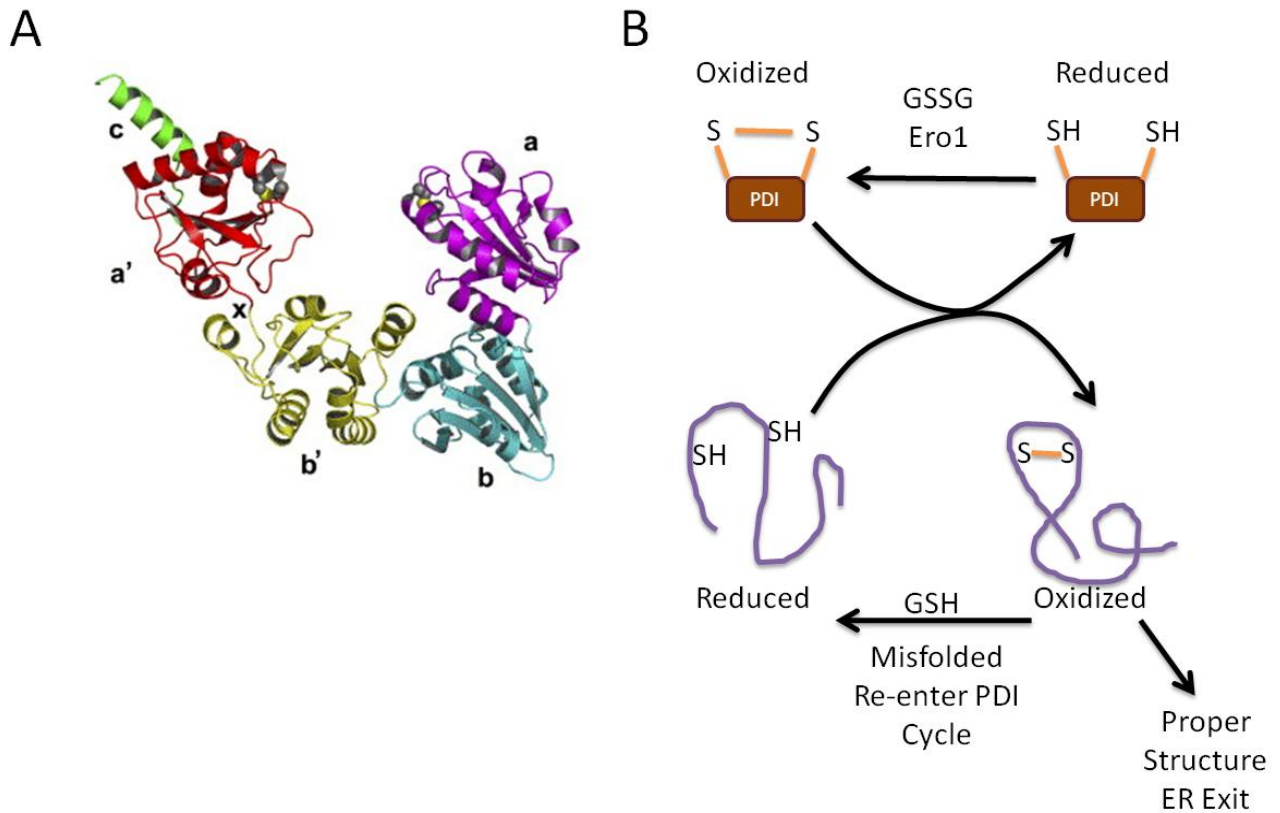
#### **1.3.2.4 Protein Disulfide Isomerase**

The ER lumen is an oxidizing environment which favors the formation of disulfide bonds (for reviews on the folding in the ER see (Tu and Weissman, 2004; van Anken and Braakman, 2005)). These bonds are important for stabilizing the folded structure of the protein, however oxidized cysteine mismatches can occur, causing the protein to achieve an aberrant conformation that may result in ER retention. A class of molecular chaperones involved in the formation of

proper disulfide bonds and thus protein folding are thiol oxidoreductases. The best characterized of these proteins are the protein disulfide isomerases (PDI). PDIs contain two active sites (a and a') with cysteine-X-X-cysteine motifs separated by two noncatalytic sites. PDI can not only assist a protein with disulfide bond formation, but can also function as a chaperone and regulate general protein folding (Figure 13) (Wang and Tsou, 1993).

The crystal structure of a yeast PDI was recently solved and the general shape of the protein is a "U", with the active sites at the tips of the "U" and the noncatalytic sites in the rigid base of the structure. There are patches of hydrophobic residues surrounding each active site which are proposed to be the substrate binding site (Figure 13) (Tian et al., 2006). The function of the PDI catalytic sites have been examined in yeast and, interestingly, while mutating both sites is lethal in a *pdi1Δ* background, the mutation of the a or a' site singly has no effect on cell growth. However, there are phenotypic differences in strains containing mutations in the a and a' site suggesting that they have different roles in the Pdi1p function. The a site mutants, but not a' site mutants exhibit reduced CPY maturation, a protein that is dependent upon proper disulfide bond formation for ER exit, and an inability to recover from DTT treatment (Holst et al., 1997; Luz and Lennarz, 1998).

The systematic identification and correction of inappropriate disulfide bonds is vital as evidenced by the high redundancy in the PDI family members. The mammalian ER contains at least seventeen PDIs while the yeast ER contains five of these chaperones (Buck et al., 2007; van Anken and Braakman, 2005). Pdi1p is the only essential PDI in yeast, however overexpression of any of the other four PDI homologs rescues the inviable phenotype. However there are other structural differences between the PDIs in yeast. For example, of the five PDI homologs in yeast, only Pdi1p contains 2 active sites. Eug1p has two active sites however, each site contains



**Figure 13: The Structure Of PDI And Its Role In Disulfide Bond Formation**

A: The crystal structure of the yeast protein disulfide isomerase, Pdi1p. The protein has a general “U” shape with the catalytic residues (a and a’) at the tips of the “U”. The noncatalytic sites (b and b’) are located at the base of the “U”. The interior cleft has hydrophobic patches that are believed to function in the peptide binding activity of PDI, which is essential for the protein’s function as a chaperone (Structure from (Tian et al., 2006)).

B: Disulfide bond formation of target polypeptides by PDI. The cysteine residues in the a and a’ sites of PDI in the oxidized state drive the formation of disulfide bonds in the target polypeptide. If the oxidized polypeptide is in its proper structure it will exit the ER to continue on the secretory pathway. If it is misfolded it will be reduced by reduced glutathione (GSH) to reenter a folding pathway with PDI. Reduced PDI is re-oxidized through the action of Ero1p and oxidized glutathione (GSSG).

a single cysteine in each active site that allows the protein to break previously formed disulfide bonds, but prevents the PDI from participating in catalyzing new intramolecular disulfide bond formation (Tachibana and Stevens, 1992). The remaining three of the PDI homologs, Mpd1p, Mpd2p, and Eps1p, all have only one active site with the motif cysteine-X-X-cysteine (Norgaard et al., 2001).

PDIs have been found to also function in targeting misfolded proteins for retrotranslocation and ERAD (Forster et al., 2006; Wahlman et al., 2007). For example, two thiol oxidoreductases were recently found to be involved in the removal of disulfide bonds critical for SV40 viral uncoating and retrotranslocation of viral proteins from the ER; processes essential for the infectious cycle of the virus (Schelhaas et al., 2007). The exact contribution of each individual PDI to ERAD and retrotranslocation has not been assessed. However, studies on the yeast PDI deletion mutants examining viability and CPY maturation revealed that individual PDIs do exhibit distinct phenotypic differences (Norgaard et al., 2001).

As mentioned previously, ApoB interacts with MTP, a protein complex that contains PDI as one of its subunits. The interaction of ApoB with MTP is critical for efficient translocation of ApoB into the ER lumen (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Dashti et al., 2002; Jamil, 1996; Liang and Ginsberg, 2001; Mitchell et al., 1998; Patel and Grundy, 1996; Tietge et al., 1999; Wu et al., 1996). Also, the N-terminus of ApoB is highly disulfide bonded and the formation of these bonds has been linked to efficient lipoprotein secretion (Dashti et al., 2002; Segrest et al., 1999; Wu et al., 1996). However, no experiments have directly addressed the direct contribution of PDI to ApoB ERAD.

### 1.3.2.5 p58<sup>IPK</sup>

Another ER luminal factor in the mammalian ER that regulates ApoB translocation and degradation is the J domain containing protein p58<sup>IPK</sup>. This protein is inducible by the unfolded protein response (UPR), which helps the cell respond to ER stress. A proposed role for p58<sup>IPK</sup> in the UPR is inhibition of the eIF2 $\alpha$  kinase, PERK. PERK activity is required to stall protein translation upon the induction of a UPR to prevent further overload of the ER. However, later stages of the UPR require translation of UPR specific gene products. The inhibition of PERK by p58<sup>IPK</sup> ultimately allows translation to resume and generate UPR response proteins (Yan et al., 2002). The Ron lab determined that p58<sup>IPK</sup>  $-/-$  mouse fibroblast cells accumulate a higher level of misfolded proteins when exposed to prolonged stress (Oyadomari et al., 2006). The Hegde lab observed a 40-50% reduction in protein synthesis in p58<sup>IPK</sup>  $-/-$  cells, which correlates well with a role for p58<sup>IPK</sup> in PERK inhibition. Both groups found that p58<sup>IPK</sup> mutant cells were more sensitive to ER stress in spite of the lower substrate burden (Oyadomari et al., 2006; Rutkowski et al., 2007).

Most of the p58<sup>IPK</sup> in the cell is associated with the rough ER and only protein substrates that are co-translationally degraded such as ApoB and a VCAM-1 are stabilized in p58<sup>IPK</sup>  $-/-$  cells (Oyadomari et al., 2006). Although originally proposed to be cytosolically exposed and involved in recruiting Hsp70 to target co-translational substrates for degradation, it was subsequently revealed that p58<sup>IPK</sup> is in the ER lumen and interacts with BiP (the mammalian Kar2p homolog) (Oyadomari et al., 2006; Rutkowski et al., 2007). Like Sec63p, the Hegde lab proposes a role for p58<sup>IPK</sup> to function with BiP and regulate protein translocation; however neither ApoB translocation efficiency nor degradation were examined in their study (Rutkowski et al., 2007).

## **1.4 MOLECULAR CHAPERONES AND APOLIPOPROTEIN B ER-ASSOCIATED DEGRADATION**

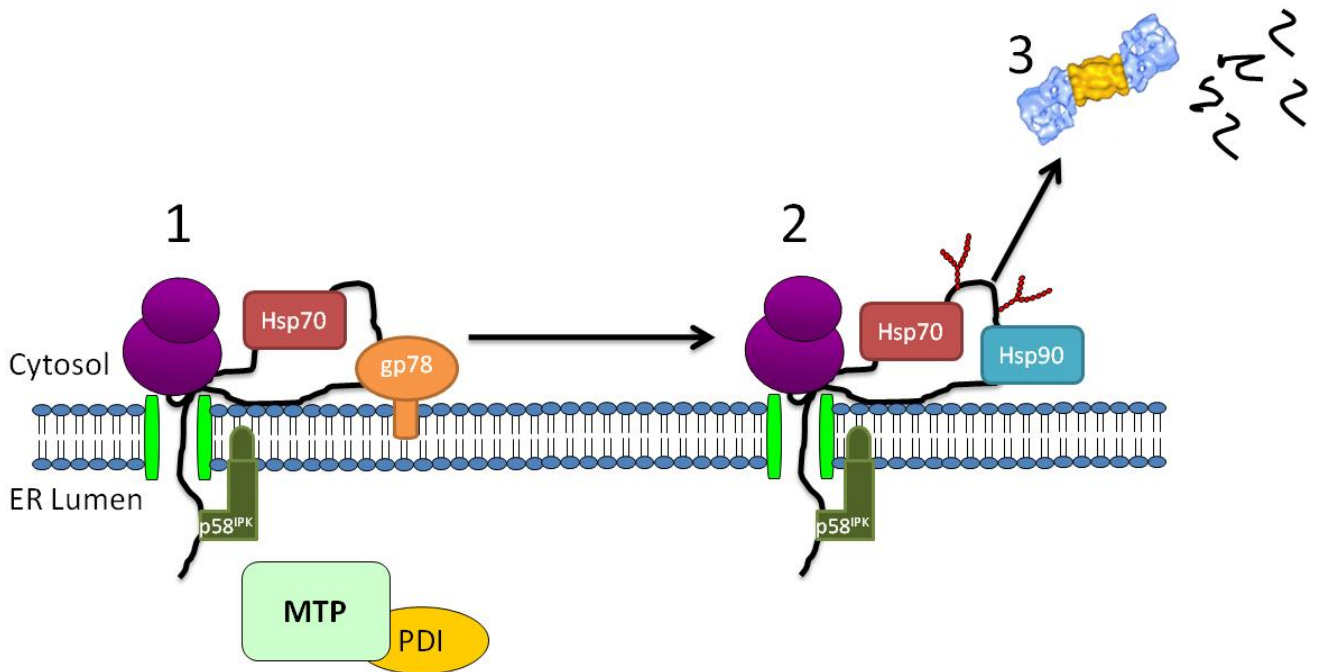
As mentioned previously, ApoB is a co-translational target for ERAD during cellular conditions that slow the translocation of the polypeptide into the ER (see section 1.1.3). The currently model for ApoB maturation includes chaperones that have been shown to directly impact ApoB biogenesis (Hsp70 and Hsp90) and chaperones that have merely been identified as interacting partners. In the next few paragraphs, I will summarize the chaperones and UPP factors that are known to interact with ApoB and the current model for how these chaperones impact ApoB secretion and ERAD (Table 3 and Figure 14).

As ApoB is synthesized by the ribosome it is co-translationally translocated into the ER with the assistance of the lipid loading protein complex, MTP. When core lipids are abundant and MTP is functional, ApoB fully translocates into the ER to form a primordial lipoprotein. While in the ER, ApoB is reported to interact with the ER luminal chaperones BiP, p58<sup>IPK</sup>, ERp72, GRP94, calreticulin, PDI, cyclophilin B, and calnexin (Adeli et al., 1997; Benoist and Grand-Perret, 1997; Linnik and Herscovitz, 1998; Oyadomari et al., 2006; Tatu and Helenius, 1999; Zhang and Herscovitz, 2003). p58<sup>IPK</sup> is proposed to function with BiP in assisting with the targeting of protein substrates to ERAD and in the absence of the J domain containing protein, p58<sup>IPK</sup> ApoB is stabilized (Oyadomari et al., 2006; Rutkowski et al., 2007). Only calnexin,



**Table 3: Molecular Chaperones And UPP Components Reported To Interact With ApoB**

<b>Protein</b>	<b>Description</b>	<b>Interaction with ApoB</b>
<b>MTP</b>	ER luminal lipid transfer protein	Required for efficient translocation and formation of primordial lipoprotein
<b>PDI</b>	ER luminal chaperone involved in proper disulfide bond formation	Immunoprecipitates with ApoB and is required for translocation and secretion
<b>BiP</b>	ER luminal Hsp70	Immunoprecipitates with ApoB in both ER and Golgi Fractions
<b>ERp72</b>	ER luminal thioredoxin (PDI homolog)	Immunoprecipitates with ApoB in both ER and Golgi fractions
<b>ERp60</b>	ER luminal thioredoxin like protein and putative protease (PDI homolog)	Immunoprecipitates with ApoB
<b>GRP94</b>	ER luminal Hsp90	Immunoprecipitates with ApoB in both ER and Golgi fractions
<b>Calreticulin</b>	ER luminal chaperone involved in lectin cycle	Immunoprecipitates with ApoB in both ER and Golgi fractions and is required for ApoB secretion
<b>Calnexin</b>	ER luminal chaperone involved in lectin cycle	Immunoprecipitates with ApoB and is required for ApoB secretion
<b>p58<sup>IPK</sup></b>	ER luminal J domain protein that interacts with BiP	Required for efficient degradation of ApoB
<b>Cyclophilin B</b>	ER luminal chaperone that function as peptidyl- prolyl- cis-trans- isomerases	Immunoprecipitates with ApoB in both ER and Golgi fractions
<b>Hsp70</b>	Cytosolic Hsp70	Targets inefficiently translocated ApoB for polyubiquitination and proteasomal degradation
<b>Hsp90</b>	Cytosolic Hsp90	Targets inefficiently translocated ApoB for proteasomal degradation
<b>gp78</b>	Cytosolic E3	Assists with polyubiquitination and targeting of ApoB for proteasomal degradation



**Figure 14: Factors Known To Impact ApoB ERAD**

The translocation of ApoB is assisted by the MTP-PDI protein complex and p58<sup>IPK</sup>. Inefficient ApoB translocation results in a bitopic orientation in the translocon due to poor lipid transfer by MTP. This exposes cytosolic domains of ApoB that are bound by Hsp70. The Hsp70 bound ApoB is subsequently polyubiquitinated and the E3, gp78, has been shown to contribute to this process. The polyubiquitinated ApoB is bound by both Hsp70 and Hsp90 and can be targeted to the proteasome for degradation.

calreticulin, and PDI have been shown to be important for ApoB secretion. This was accomplished by examining of cells treated with compounds that inhibit the function of the proteins or by ApoB mutational analysis (Dashti et al., 2002; Segrest et al., 1999; Tatu and Helenius, 1999; Wu et al., 1996). The other chaperones have been only been detected in ApoB immunoprecipitates. Interestingly, GRP94, ERp72, calreticulin, BiP and cyclophilin B were also found associated with ApoB in Golgi fractions, suggesting that they travel with ApoB to the Golgi and further assist in the transition from the primordial lipoprotein to the mature VLDL particle (Zhang and Herscovitz, 2003).

When core lipids are not abundant or in the presence of a non-functional MTP, the co-translational translocation of ApoB into the ER stalls (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Fisher et al., 1997; Liao et al., 1998; Sakata and Dixon, 1999; Yeung et al., 1996). The cytoplasmically exposed loops of ApoB that form by continued translation are bound by the cytosolic chaperones Hsp70 and Hsp90 (Fisher et al., 1997; Gusarova et al., 2001). Subsequently, ApoB is recognized by gp78, an E3 ubiquitin ligase, polyubitinated, and degraded by the proteasome (See Figure 14 and Table 3) (Benoist and Grand-Perret, 1997; Du et al., 1999; Fisher et al., 1997; Gusarova et al., 2001; Hrizo et al., 2007; Liang et al., 2003; Liao et al., 1998; Linnik and Herscovitz, 1998; Sakata and Dixon, 1999; Tatu and Helenius, 1999; Yeung et al., 1996; Zhang and Herscovitz, 2003; Zhou et al., 1998).

Currently, the contributions of many of the ApoB associated ER lumenal factors to the co-translational ERAD of ApoB have not been examined. In addition, the cell contains many E3s which can function redundantly (Nakatsukasa et al., 2008); therefore additional E3 ubiquitin ligases may participate in ApoB turnover. Also, an E2 for ApoB has not been identified. Finally, in the previous sections I mentioned that many chaperones and co-chaperones regulate

Hsp70 and Hsp90 function. The roles of these cooperating factors, such as Hsp40s, p23, and Hop, on ApoB degradation are unknown.

The goal of my dissertation is to examine the molecular mechanism of ApoB ERAD. In order to examine how chaperones contribute to ApoB biogenesis and degradation I utilized a previously described *in vitro* degradation assay and developed a yeast expression system for ApoB29, an isoform of the human ApoB protein (Gusarova et al., 2001). I also employed both systems to examine the interactions of ER luminal and cytosolic chaperones and co-chaperones with ApoB. Finally, I confirmed select results obtained with the *in vitro* and *in vivo* yeast systems by examining ApoB degradation and secretion in a rat hepatoma cell line.

## **2.0 CHARACTERIZATION OF APOLIPOPROTEIN B ERAD UTILIZING A YEAST EXPRESSION SYSTEM**

As described in section 1.4, the contributions of cytosolic and ER luminal molecular chaperones to ApoB degradation is not fully understood. Examining the chaperones that facilitate ApoB secretion and degradation in mammalian systems due to the redundancy of the molecular chaperone composition of the cells has been difficult. In addition, the modulation of chaperone expression levels by overexpression vectors and siRNA and the inhibition of chaperone activity with compounds can induce the UPR and result in up-regulation of other chaperones (Maloney et al., 2007; Suzuki et al., 2007). This may give rise to secondary effects.

Importantly, ERAD is a highly conserved process among eukaryotes. Many yeast strains containing deletions or mutations of molecular chaperones are already available, and these strains have been shown to exhibit ERAD defects for various mammalian substrates (for recent reviews see (Brodsky, 2007; Nishikawa et al., 2005)). Previous work by Viktoria Gusarova in the Fisher laboratory at the New York University School of Medicine demonstrated that yeast cytosol is sufficient to support the proteasomal degradation of ApoB in vitro (See Section 3.0) (Gusarova et al., 2001). Furthermore, Hsp70 and Hsp90 function are required for ApoB degradation in both mammalian cell culture and the in vitro yeast system (Fisher et al., 1997; Gusarova et al., 2001; Zhang et al., 2001). Currently, Hsp70, Hsp90, p58<sup>IPK</sup> and the E3, gp78, are the only factors known to be important for targeting ApoB for co-translational degradation

(Fisher et al., 1997; Gusarova et al., 2001; Liang and Ginsberg, 2001; Oyadomari et al., 2006; Zhou et al., 1995).

In order to examine the chaperone and co-chaperone requirements for ApoB ERAD I developed an ApoB expression system in yeast. In this section, I will present data that indicates that ApoB is ER localized when expressed in yeast and requires components of the UPP for degradation. It should be noted that in some cases, heterologous protein expression in yeast and overexpression of yeast proteins has physiological implications that includes a change in intracellular morphology (Becker et al., 1999; Umebayashi et al., 1997; Wright et al., 1988; Zhang et al., 2001). For example, the mammalian protein CFTR does not traffic to its functional location, the plasma membrane, when expressed in yeast and causes an expansion of the ER membrane (Huang et al., 1996; Zhang et al., 2001). While ApoB is secreted from hepatic and intestinal cells, I did not observe secretion of ApoB from yeast. The missing components of the secretory pathway required for trafficking of CFTR from the ER to the plasma membrane and the secretion of ApoB in yeast are not known. However, mammalian cells do contain unique factors that impact the secretion of ApoB such as p58<sup>IPK</sup>, an ER luminal protein that does not have a homolog in yeast (Oyadomari et al., 2006). Therefore it is possible that other mammalian factors that are absent from yeast may be required for the efficient secretion of ApoB. In addition, the glycosylation of proteins in yeast is similar, but not identical to glycosylation in mammalian cells. For example, yeast utilize sugar chains containing mannose residues for N-linked glycosylation in the ER while the mammalian sugar chains contain different sugars (For a recent review see (Chiba and Jigami, 2007)). Regardless, I was able to identify the cytosolic Hsp110, Sse1p, as a modulator of ApoB ERAD. Unlike the previously identified cytosolic pro-

degradation chaperones, Hsp70 and Hsp90, Sse1p helps stabilize ApoB (Gusarova et al., 2001; Zhou et al., 1995).

Sse1p is reported to function in heterodimeric complexes with two different Hsp70s, Ssa1p and Ssb1p (Shaner et al., 2005; Yam et al., 2005). As Ssa1p is already known to function as a pro-degradation chaperone for ApoB, I examined the role of the other Sse1p associated Hsp70, Ssb1p, by cycloheximide chase analysis. Ssb1p is a member of the ribosome associated complex and associates with nascent polypeptides as they exit the ribosome (Gautschi et al., 2001; Gautschi et al., 2002; Pfund et al., 1998). This Hsp70 was an intriguing target as ApoB is targeted for degradation while still being synthesized by the ribosome (Chen et al., 1998; Gusarova et al., 2001; Liang et al., 1998; Liang et al., 2003; Liao et al., 1998; Zhou et al., 1995). In addition, I investigated the requirements for Sse1p ATPase activity on ApoB stabilization. Finally, the other Hsp70 NEFs, Snl1p and Fes1p, were examined for a role in ApoB degradation.

As mentioned previously, genetic information has linked Sse1p function to Ydj1p and to the Hsp90 complex (Goeckeler et al., 2002). While Hsp90 is known to be a pro-degradation factor for ApoB, the interaction of ApoB with other members of the Hsp90 complex and their roles in ApoB degradation are unknown. In this chapter I have included data that indicate that the cytosolic Hsp40s, Ydj1p and Hlj1p, are pro-degradation factors for ApoB. I subsequently examined the interaction of ApoB with select members of the Hsp90 complex to determine if all of the Hsp90 complex members form stable interactions with ApoB and if the ApoB interactors are required for ApoB degradation.

## **2.1 MATERIALS AND METHODS**

### **2.1.1 Yeast Strains, Plasmids, And Antisera**

Yeast strains (Table 4) were grown under standard conditions at 30°C unless otherwise noted, and established media and manipulations were used (Adams et al., 1998). Antibodies used in this study are listed in Table 5.

To express ApoB29 in yeast, plasmid pSLW1 was first constructed using pJJB20 (kindly provided by Dr. R. Fuller, University of Michigan), which contains a pBM258 backbone with amino acids 1-100 from the yeast mating factor alpha 1 locus inserted into the BamHI and SalI restriction sites (Zhang et al., 1994). Next, a triple hemagglutinin (HA) tag (YPYDVPDYA) was PCR amplified with a 5' XbaI, an internal ClaI, a 3' SalI restriction site, and tandem stop codons (5'-TAA TGA-3'), and was inserted into pJJB20 at the XbaI and SalI sites to form plasmid pSLW1 (Table 6). Next, ApoB29 was amplified from the SP6-ApoB48 plasmid (Gusarova et al., 2001) by PCR with the following primers: (forward) 5'-ATT GCC AGC ATT GCT AAA GAA GAA GGG GTA TCA CTA CTC AAG AGG AAA ATG TCA GCC TGG TCG TC-3' and (reverse) 5'-GGG ATA GCC CGC ATA CTC AGG AAC ATC GTA TGG GTA ATC GAT ACT GTA GGA GGC GGA CCA GTT GCT-3'. Finally, ClaI and XbaI digested pSLW1 and the ApoB29 PCR product were co-transformed into yeast strain W3031b and colonies containing the gap-repaired pSLW1 plasmid with the ApoB29 insert were selected by growth on synthetic complete medium lacking uracil. Plasmid DNA from yeast harboring the pSLW1-B29 plasmid (Figure 15A, Table 6) was prepared and the integrity of the plasmid was confirmed by sequence analysis. All other plasmids used in this study are listed in Table 6.



### 2.1.2 Cycloheximide Chase Analysis Of ApoB ERAD

To measure ApoB29 ERAD in yeast, cells transformed with pSLW1-B29 (see above) were grown to logarithmic phase ( $OD_{600} = 0.4-1.0$ ) overnight at 26°C in synthetic complete medium lacking uracil but supplemented with glucose to a final concentration of 2%. To obtain maximal expression of ApoB29, the cells were harvested and resuspended to an initial concentration of 0.5  $OD_{600}/ml$  in complete medium (Yeast extract-Peptone) containing galactose at a final concentration of 2% and were grown at 30°C for 5h. To monitor ApoB degradation, protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50 $\mu g/ml$ , 2 ODs of cells were harvested at the indicated time points, and total protein was precipitated as previously described (Zhang et al., 2001). The Supersignal West Pico Chemiluminescent Substrate (Pierce) was utilized for immunoblots of loading controls and the Supersignal West Femto Maximum Sensitivity Substrate (Pierce) was used for anti-HA and anti-ApoB immunoblots. The signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D (v3.6) software (Rochester, NY). The cycloheximide chase analysis of CFTR-HA and Ste6p\*-HA and the pulse chase analysis of ppof $\Delta$ G-HA and CPY\*-HA were performed as previously described (Kim et al., 2005; Loayza et al., 1998; Ng et al., 2000; Zhang et al., 2001). Previous studies on ERAD in yeast have failed to uncover a difference in the chaperone-dependence on the degradation of ER-associated proteins when either cycloheximide or metabolic labeling followed by a methionine chase was employed (J.L.B., unpublished data.) For all cycloheximide chase analyses, each time point was normalized to the L3 or Sec61p loading control and the relative amount of ApoB was calculated by dividing the signal at each time by the value at  $t = 0$ . For oxidative stress experiments, diamide (Sigma) was added to the media at a final concentration of 1mM.

### 2.1.3 Biochemical And Immunological Methods

ApoB29 expression was induced as above and a total of 100 ODs of cells were harvested and fractionated as previously published (Kabani et al., 2003). To assess whether ApoB29 was carbonate-extractable, ~40µg of yeast lysate prepared as described for the fractionation analysis were treated with 100mM NaCO<sub>3</sub>, pH 11.5 (Kabani et al., 2003). The ApoB sucrose gradient flotation assay was performed as published (Zhang et al., 2001).

For ApoB interaction studies, cell extracts were prepared by glass bead lysis from 100 ODs of yeast grown as described above and immunoprecipitations were conducted in the presence of 20mM NaMoO<sub>4</sub> (Yam et al., 2005) using anti-Sse1p, anti-Ssa1p, anti-Ssb1p, anti-Sec61p, or anti-Sec63p antiserum (see above). For ApoB immunoprecipitation experiments with the anti-HA resin (Roche), cell extracts from 100 ODs of yeast were prepared by 3, 5 min agitations with glass beads in Roche lysis buffer (50mM Tris, pH 7.5, 25mM NaCl, 0.1% Nonidet P40, 20mM NaMoO<sub>4</sub>) with protease inhibitors (0.25mM MG132, 1mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin A). Next, 1mg of cell extract was incubated overnight at 4°C with anti-HA resin or sepharose 6B beads that were used as a negative control (Sigma). Unless otherwise noted, the beads were washed twice with Roche lysis buffer containing 150mM NaCl and 300mM NaCl respectively. In addition to the Roche buffer 150mM and 300mM NaCl washes, ApoB-Hsp90 complex immunoprecipitations were also conducted using two wash buffers containing 1X TBS (25mM Tris, pH 7.4, 3mM KCl, 140mM NaCl) with 0.5% Tween20 and 1% Tween20 respectively. The isolated protein precipitates were resolved by SDS-PAGE and the relevant proteins were identified by immunoblot analysis as described above.

Indirect immunofluorescence was performed as previously described (Coughlan et al., 2004). ApoB29 expression was induced as described above for 2 and 4h. Subsequently, the

cells were fixed in 3.7% formaldehyde and treated with 20 $\mu$ g/mL zymolase for 45min at 37°C. The cells were then incubated with the primary antibodies: anti-HA, 1:250 (Roche, anti-mouse) and anti-Kar2p (Brodsky and Schekman, 1993), (anti-Rabbit) 1:250 overnight at 4°C. The cells were then incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse 1:250, Alexa Fluor 568 goat anti-rabbit 1:250; Molecular Probes) for 2h at room temperature.

#### **2.1.4 Detecting Polyubiquitinated ApoB In Yeast**

Polyubiquitinated proteins were detected through the use of a modified protocol (Ahner et al., 2007). In brief, 50 ODs of yeast transformed with pSLW-B29 and a pSM989 (a copper inducible myc-tagged ubiquitin construct (Ellison and Hochstrasser, 1991)) were obtained from an overnight culture in selective media (-Ura-Trp+Glu). ApoB expression was induced in YP-GAL for 4h and then Ub-Myc expression was induced with the addition of CuSO<sub>4</sub> at a final concentration of 100  $\mu$ M for 1h. The cells were glass bead lysed in Roche lysis buffer with protease inhibitors as described above with the addition of 10 mM *N*-ethylmaleimide (NEM) to the buffer. Next, ~1mg of the cell extracts were incubated for 4h at 4°C with anti-HA resin or sepharose 6B beads that were used as negative control (Sigma). The beads were washed three times with a buffer containing 1% Triton X 100, 0.2% SDS, 150mM NaCl, 5mM EDTA, and 50mM Tris, pH 7.4 treated with fresh 10mM NEM. The isolated protein precipitates were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblot. ApoB was detected on the immunoblot as described above in section 2.1.2. To detect ubiquitination, the immunoblots were boiled for 20 min in double distilled H<sub>2</sub>O, blocked in Blotto, and ubiquitin was then detected with anti-Myc (a kind gift from Susan Michaelis at Johns Hopkins University, 1:5000).

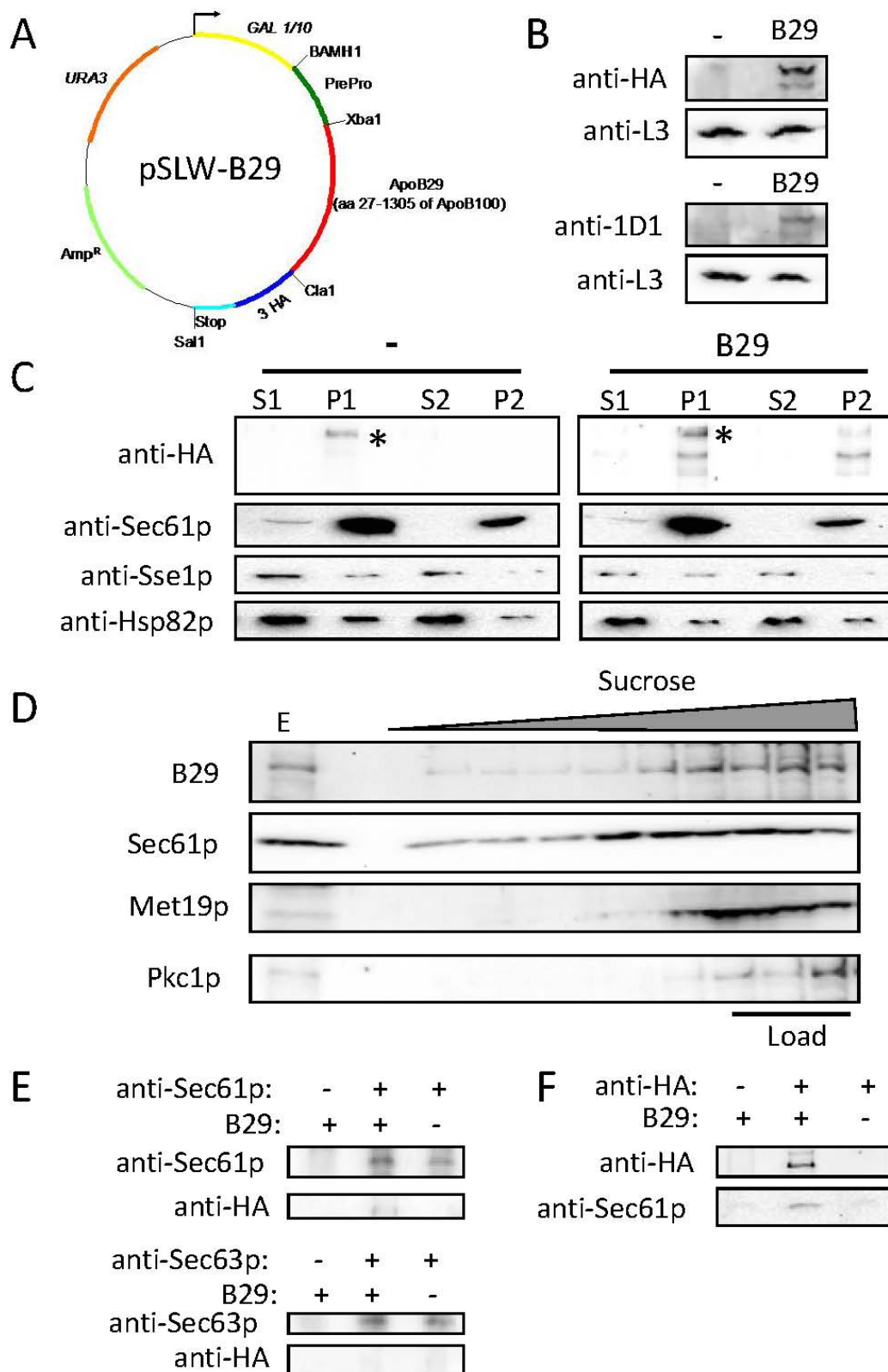
## 2.2 RESULTS

### 2.2.1 ApoB Can Be Expressed In Yeast And Is Targeted To The ER

In order to develop a genetic system in which other components required for ApoB biogenesis can be isolated or examined, a yeast ApoB expression system was developed. For this purpose, a galactose-inducible yeast expression vector was constructed to produce an HA-tagged ApoB isoform that is ~29% of the size of full-length ApoB (Figure 16A; pSLW1-B29). In mammalian cells, ApoB29 forms a lipoprotein particle and is the shortest form of ApoB that matures and traffics normally through mammalian cells (Segrest et al., 2001). Because of yeast codon bias (Figure 15), the ApoB signal sequence was replaced with the signal sequence and pro-region from yeast pre-pro- $\alpha$  factor. This pre-pro sequence was chosen because it was used previously to express the  $\beta$ -Amyloid Precursor Protein (APP) in yeast (Zhang et al., 1994).

Under induction conditions (see Methods and Materials), the pSLW1-B29 plasmid (Figure 16A) directed the expression of a protein of the correct molecular mass (~160kDa) and that was detected by both anti-HA and anti-ApoB antibodies (Figure 16B). The localization of the expressed protein was then examined by sub-cellular fractionation. As shown in Figure 16C, ApoB29 was present in the pellet fractions (P1 and P2)—as was Sec61p, a component of the ER translocon—suggesting membrane-association. In contrast, both Sse1p and Hsp82p, which loosely associate with the ER, were detected primarily in the second supernatant (S2). To confirm that ApoB29 associates with membranes in yeast, cell extracts were mixed with a dense sucrose solution and over-laid with sucrose solutions of lower density. Following high-speed





**Figure 16: ApoB29 Is Membrane and Translocon Associated in yeast**

A) Plasmid map of pSLW1-B29, a galactose inducible, multicopy yeast ApoB29 expression vector.

B) Both anti-HA and anti-ApoB (1D1) antibodies detect ApoB29, a ~160kDa protein, expressed in yeast. Blots containing extracts from control (-) or ApoB29 expressing (B29) cells were also probed for a ribosomal protein, L3, as a loading control.

C) A fraction of the ApoB29 floats with membranes after differential centrifugation of lysates from ApoB29 expressing (B29) or control cells (-). Note the slower migrating band that nonspecifically cross-reacts with the HA antibody (marked with \*). S1 = 16,000g supernatant, P1 = 16,000g pellet, S2 = 150,000g supernatant, P2 = 150,000g pellet. Protein levels were normalized by SDS-PAGE and Coomassie Brilliant blue staining prior to western blot analysis. The material that fails to float (~50-60% of the total ApoB protein) may represent the aggregated material shown in Figure 18.

D) ApoB29 and the ER membrane associated protein, Sec61p, migrate to a lower sucrose density when extracts from ApoB expressing cells are layered in a sucrose gradient. The soluble cytosolic proteins glucose-6-phosphate dehydrogenase (Met19p) and protein kinase C (Pkc1p) remain in the fractions where the extracts were loaded into the gradient. The lane marked "E" indicates 1% of the input (load). A similar flotation pattern was observed for Sec61p, Met19p, and Pkc1p in cells transformed with the empty vector control (data not shown). ~70% of the total Sec61p in the extract floated to fractions of lower sucrose density, while ~42% of ApoB in the extract was found in the lower sucrose density fractions.

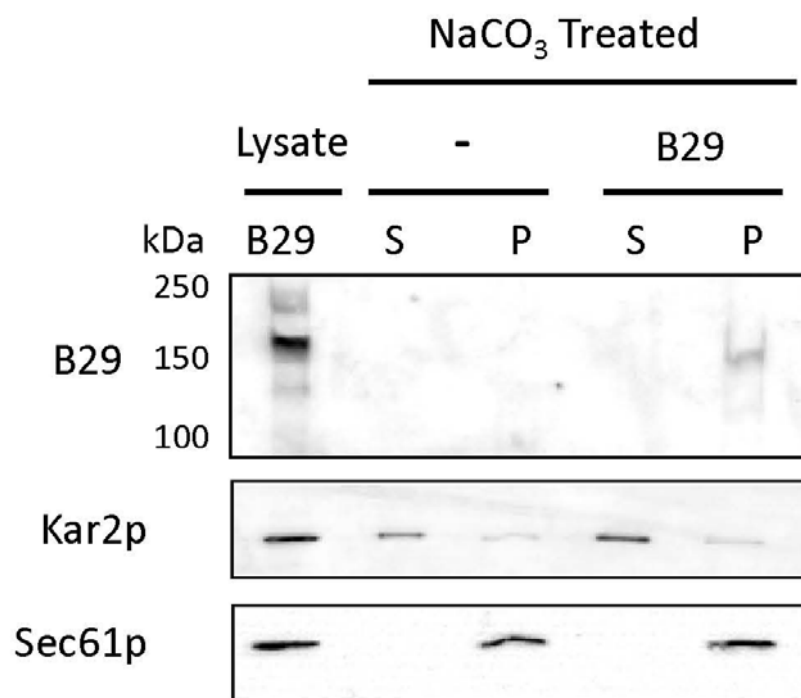
E) ApoB29 co-precipitates with Sec61p. Cell extracts were prepared from cells transformed with a vector control or pSLW1-B29 (B29) and were mock treated or were incubated with anti-Sec61p or anti-Sec63p and protein A sepharose. The total proteins in the precipitates were resolved by SDS-PAGE and were immunoblotted with the indicated antisera.

F) In the reciprocal experiment, Sec61p co-precipitate with ApoB29. Cell extracts were prepared and incubated with anti-HA resin ("+") or sepharose 6B beads ("-"). The precipitates were immunoblotted with the indicated antisera.

centrifugation, much of the ApoB29 was found in fractions of lower sucrose density with Sec61p (Figure 16D). Note that the cytoplasmic proteins, glucose 6-phosphate dehydrogenase (Met19p) and protein kinase C (Pkc1p), remained in the load fractions and did not float. In addition, an intimate membrane-association of ApoB29 in yeast was established by carbonate extraction (Figure 17). Finally, when the localization of ApoB was examined by indirect immunofluorescence, some overlap with the ER was detected by co-localization with Kar2p staining (Figure 18). However, it should be noted that there are large aggregates of protein in the cytoplasm that do not overlap with the ER. This may represent the portion of ApoB that cannot be degraded in cycloheximide chases (Only about 40-60% of the total ApoB pool can be degraded in a cyclohexamide chase in a wildtype yeast cell see Figure 20 below).

In order to assess if the ApoB isoform expressed in yeast interacted with the translocon, as observed in mammalian cells (Chen et al., 1998; Mitchell et al., 1998; Pariyarath et al., 2001), I employed non-denaturing conditions to immunoprecipitate Sec61p, and probed the precipitate to determine if ApoB29 was present. ApoB29 was detected in the precipitate with Sec61p, suggesting that the protein associates with the translocon (Figure 16E). Correspondingly, when ApoB29, which is HA tagged, was immunoprecipitated with anti-HA resin, Sec61p was found in the precipitate (Figure 16E). In contrast, ApoB29 was absent from the precipitate when Sec63p, another integral membrane protein in the ER, was immunoprecipitated (Figure 16E). These data indicate that ApoB29 is targeted to the ER and interacts with the translocon during its biogenesis in yeast.





**Figure 17: ApoB29 Is Carbonate Inextractable**

A total of 40µg of cell extract from yeast transformed with pSLW1 or pSLW1-B29 were treated with 100 mM sodium carbonate, pH 11.5. The pellet and supernatant from a 230,000g centrifugation of the cell extracts were analyzed by western blotting. The ER-luminal Hsp70 chaperone, BiP, was used as a control and was found in the supernatant, as predicted. In contrast, Sec61p was exclusively in the membrane fraction.

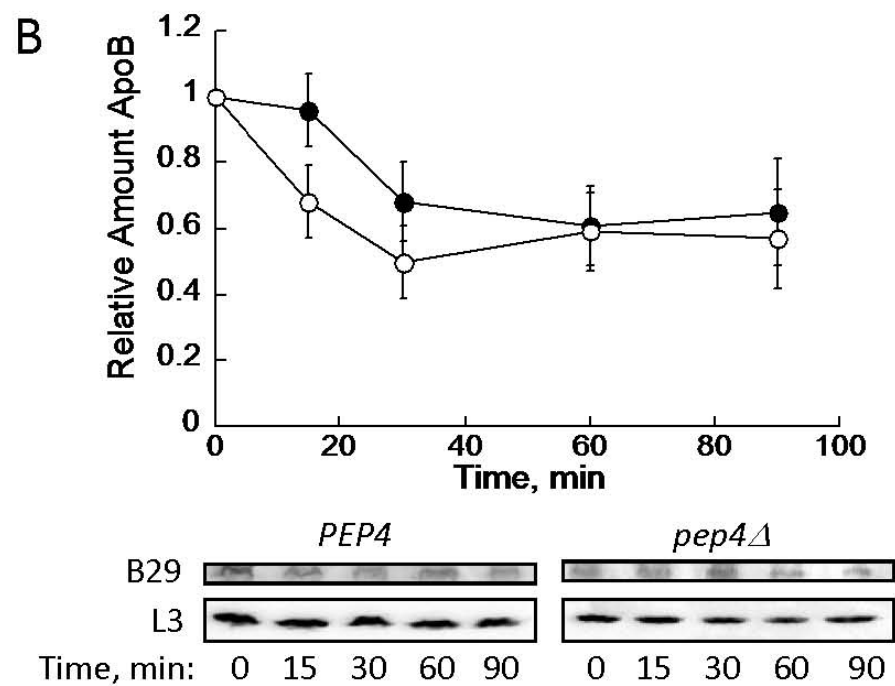
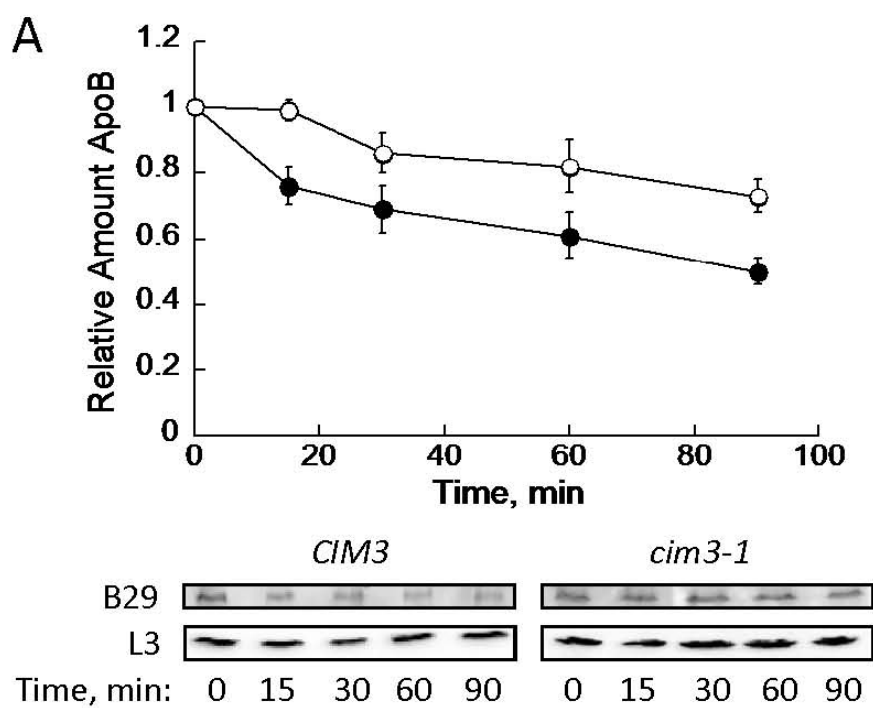


### 2.2.2 ApoB Is Degraded By The Proteasome And Requires Components Of The UPP For Its Degradation In Yeast

ApoB degradation depends upon the UPP both in vitro and in mammalian cells. To determine if the degradation of ApoB29 in yeast similarly required components of the UPP, cycloheximide chase analyses were performed to evaluate ApoB29 degradation in strains with mutations in UPP-encoding genes.

I first tested the contribution of *CIM3*, which encodes one of six AAA ATPases in the 19S regulatory particle of the 26S proteasome (Rubin et al., 1998). In *cim3-1* cells, ubiquitinated substrates are stabilized (Ghislain et al., 1993) and the ERAD of a mutant form of carboxypeptidase Y (CPY\*) and pro-alpha factor are slowed (Lee et al., 2004; Taxis et al., 2003). As displayed in Figure 19A, ApoB29 was significantly stabilized in the *cim3-1* strain. ApoB29 ERAD was also abrogated in strains containing mutations in *DOA10HRD1*, *UFD1*, *CDC48*, *UBC6UBC7*, and *UBC7* each of which are known to compromise proteasome activity and/or ERAD to varying extents (Figure 20) (Nishikawa et al., 2005). In contrast, ApoB turnover was unaltered in yeast lacking *UBC6* (another ER-associated E2; (Figure 20E)) or in cells deleted for *PEP4*, which encodes a vacuolar protease; loss of Pep4p abrogates nearly all vacuolar protease activity (Jones et al., 1982) (Figure 19B).

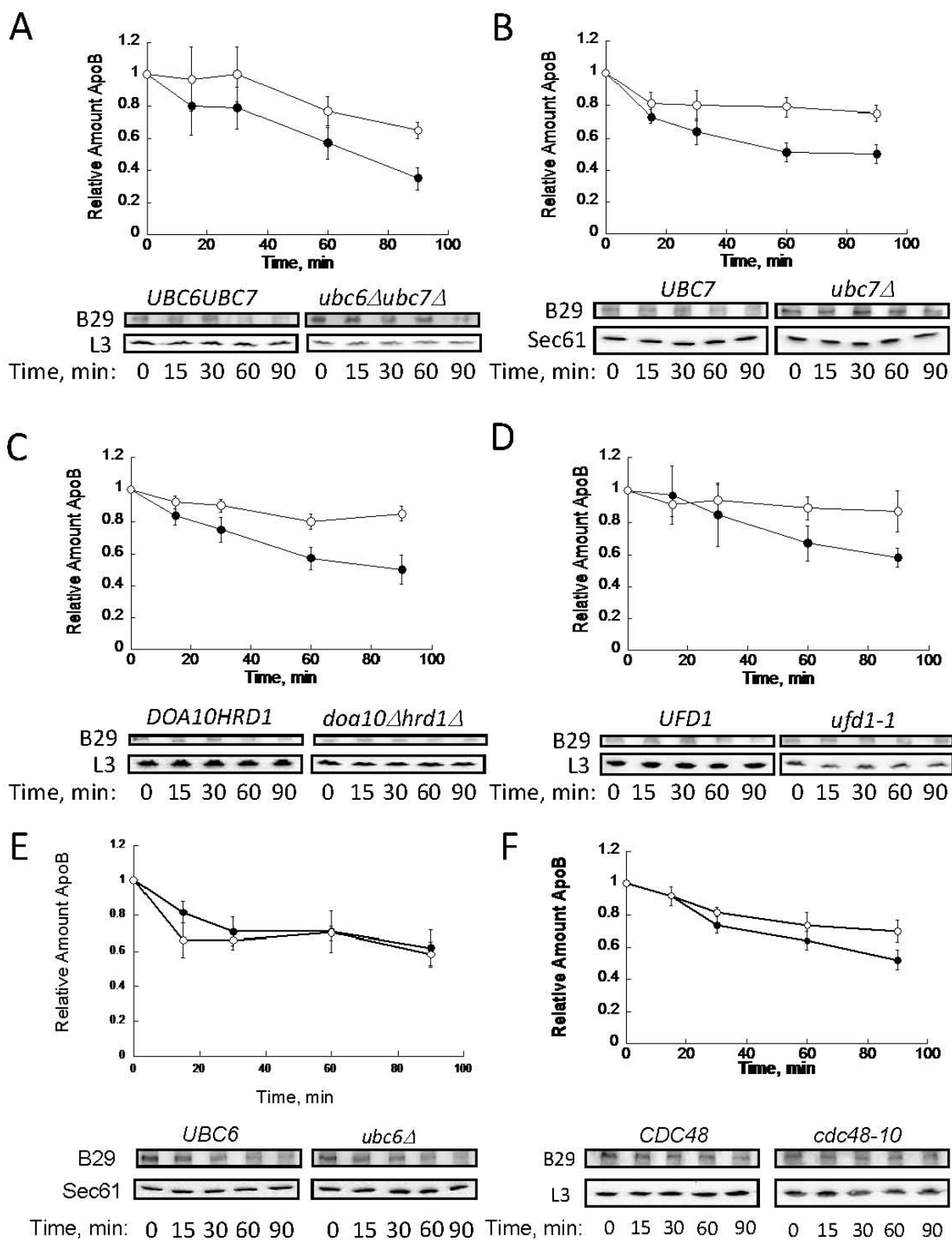
In mammalian cells, oxidative stress targets ApoB to the lysosome via the post-ER pre-secretory proteolysis pathway (PERPP) (Section 1.1.4) (Cardozo et al., 2002; Pan et al., 2004). In order to determine if ApoB is targeted to PERPP or undergoes differential degradation during



**Figure 19: ApoB29 Is Degraded By The Proteasome In Yeast.**

A) A cycloheximide chase was performed in *CIM3* (●) and *cim3-1* (○) cells transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibody and L3 was used as a loading control. For all cycloheximide chase analyses, each time point was normalized to the L3 loading control and the relative amount ApoB was calculated by dividing the signal at each time by the value at  $t = 0$ . Data represent the means from 9 independent experiments  $\pm$  SE of the means: 60 min,  $p < 0.02$ ; 90 min,  $p < 0.002$

B) A cycloheximide chase in *PEP4* cells (●) and *pep4Δ* cells (○) transformed with pSLW1-B29 was performed. Data represent the means from 6 independent experiments  $\pm$  SE of the means.



**Figure 20: ApoB Degradation Requires Components Of The UPP For Degradation In Yeast.**

- A) A cycloheximide chase was performed in *UBC6UBC7* (●) or *ubc6Δubc7Δ* cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and L3 was used as a loading control. For all cycloheximide chase analyses, each time point was normalized to the L3 loading control and the relative amount of ApoB was calculated by dividing the values at each time by the value at  $t = 0$ . Data represent the means from 6 independent experiments  $\pm$  SE of the means: 90 min,  $p < 0.005$
- B) A cycloheximide chase was performed in *UBC7* (●) or *ubc7Δ* cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. Data represent the means from 7 independent experiments  $\pm$  SE of the means: 60 min,  $p < 0.02$ ; 90 min,  $p < 0.02$
- C) A cycloheximide chase was performed in *DOA10HRD1* (●) or *doa10Δhrd1Δ* cells (○) transformed with pSLW1-B29. Data represent the means from 5 independent experiments  $\pm$  SE of the means: 60 min,  $p < 0.03$ ; 90 min,  $p < 0.002$
- D) A cycloheximide chase was performed in *UFD1* (●) or *ufd1-1* cells (○) transformed with pSLW1-B29. Data represent the means from 4 independent experiments  $\pm$  SE of the means: 90 min,  $p < 0.05$
- E) A cycloheximide chase was performed in *UBC6* (●) or *ubc6Δ* cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. Data represent the means from 6 independent experiments  $\pm$  SE of the means
- F) Before the chase the cells were shifted to 37°C and the cycloheximide chase was performed in *CDC48* (●) or *cdc48-10* cells (○) transformed with pSLW1-B29 at 37°C. ApoB29 was detected using anti-HA antibodies and L3 was used as a loading control. Data represent the means from 6 independent experiments  $\pm$  SE of the means. 90 min,  $p < 0.035$

oxidative stress, I examined ApoB degradation in two different wildtype yeast strains treated with diamide, a compound previously shown to induce ER oxidative stress in yeast (Frand and Kaiser, 1998; Wemmie et al., 1997). I observed no significant difference in ApoB turnover in the oxidatively stressed cells compared to the untreated cells (Figure 21).

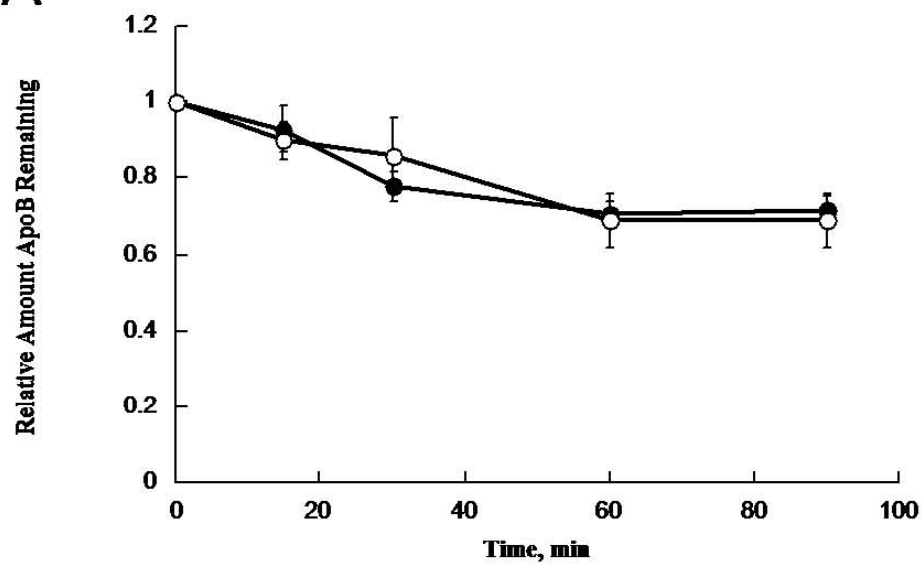
As ApoB requires components of the UPP for degradation when expressed in yeast I wanted to determine if I could detect polyubiquitinated ApoB in cell extracts. In preliminary experiments, when I utilized a previously describe myc-tagged ubiquitin overexpression system I was able to detect polyubiquitinated ApoB in yeast extracts (Figure 22) (Nakatsukasa et al., 2008). Interestingly, the smear of immunoprecipitated polyubiquitinated ApoB started below the size of the full length ApoB isoform. This suggests that proteolytic products or translation intermediates of ApoB are also polyubiquitinated. Together, the detection of polyubiquitinated ApoB coupled with the requirement for UPP components for ApoB degradation establish ApoB29 as a bona fide ERAD substrate in yeast.

### **2.2.3 Hsp110 Is A Pro-Stabilization Factor For ApoB**

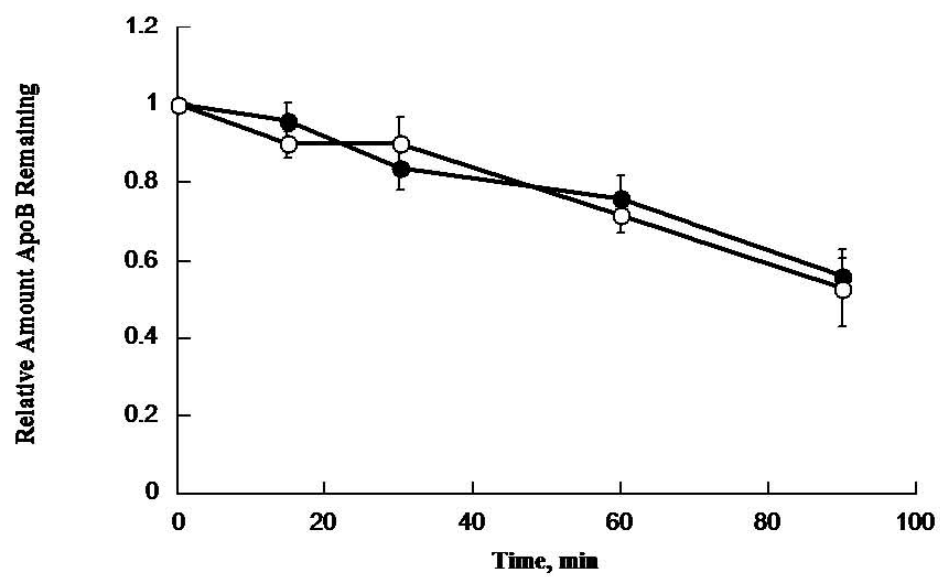
As mentioned previously, the Hsp110, Sse1p, has been reported to functionally interact with both Hsp90 and Hsp70. As these are both pro-degradation factors for ApoB I examined Hsp110 for a contribution to ApoB turnover. In contrast to results obtained in cytosol from cells expressing Hsp70 and Hsp90 mutants in which ApoB was stabilized (Gusarova et al., 2001), I found that the proteolysis of ApoB29 was enhanced in *sse1Δ* cells (Figure 23A). To determine if Sse1p and ApoB co-precipitate, extracts were prepared from *SSE1* and *sse1Δ* yeast transformed with either pSLW1-B29 or with the vector control, and a specific anti-Sse1p antiserum



**A**



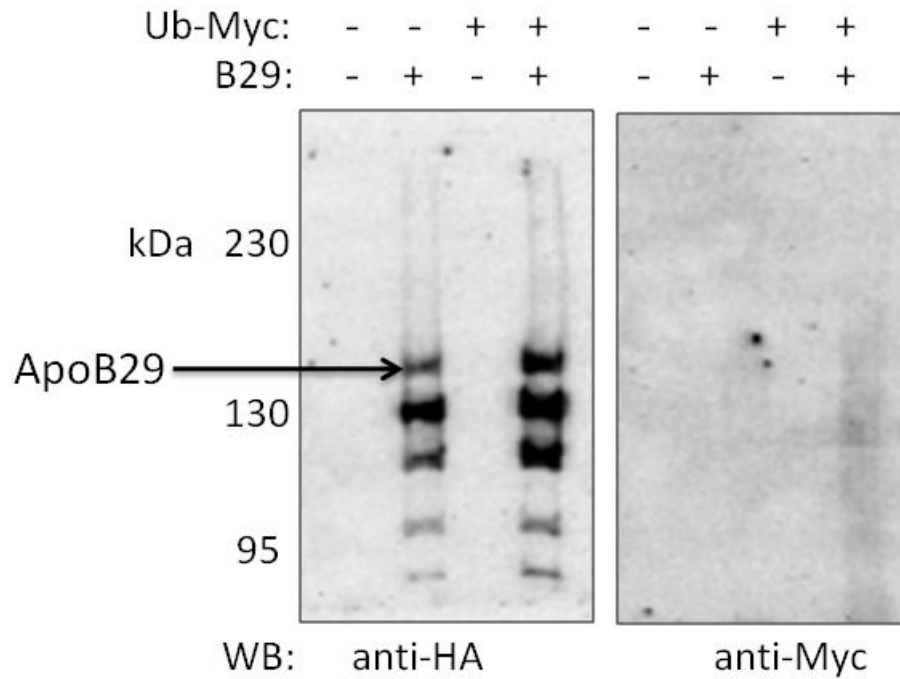
**B**



**Figure 21: Oxidative Stress Does Not Impact ApoB Degradation In Yeast**

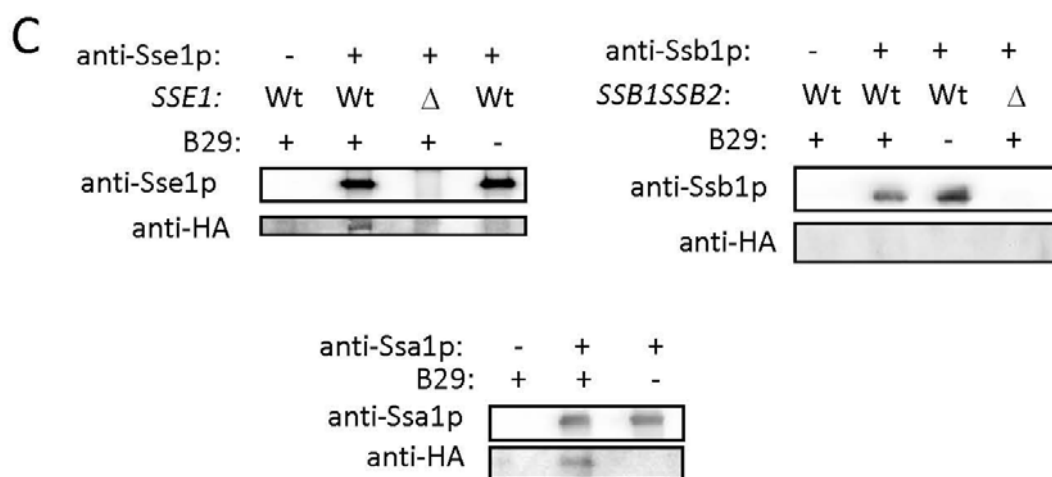
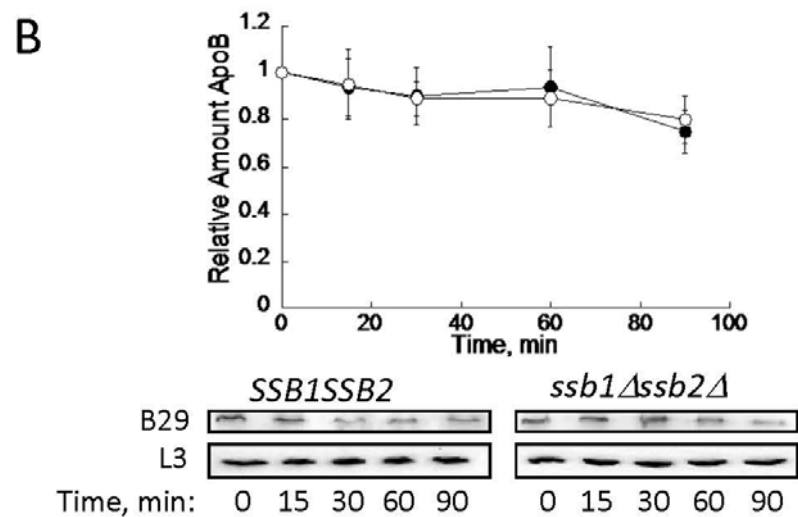
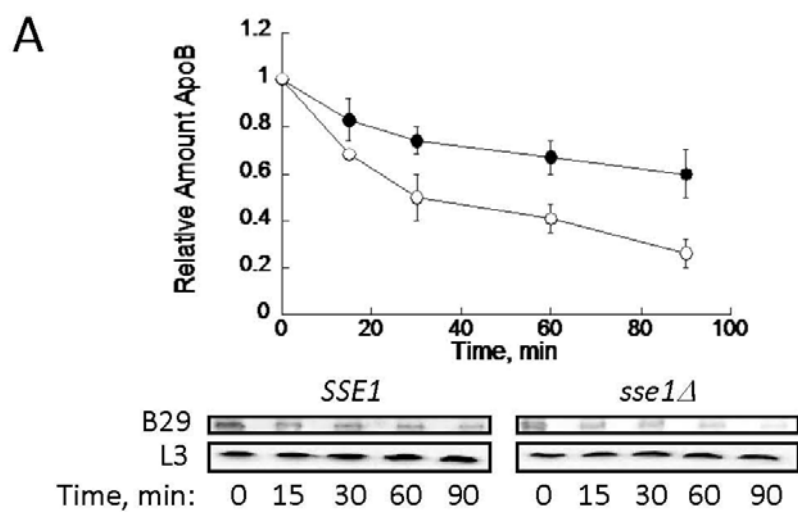
A) A cycloheximide chase was performed in untreated *SSE1* (●) or *SSE1* cells treated with 1mM Diamide (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. For all cycloheximide chase analyses, each time point was normalized to the Sec61p loading control and the relative amount of ApoB was calculated by dividing the values at each time by the value at t = 0. Data represent the means from 4 independent experiments.

B) A cycloheximide chase was performed in untreated *UBC6UBC7* (●) or *UBC6UBC7* cells treated with 1mM Diamide (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. Data represent the means from 4 independent experiments  $\pm$  SE of the means.



**Figure 22: ApoB Appears To Be Polyubiquitinated In Yeast**

ApoB29 was immunoprecipitated with anti-HA resin from ~1mg of cell extracts from yeast expressing a copper inducible Myc-tagged ubiquitin construct (Ub-Myc, +) or an empty vector control (-). In these preliminary experiments, ApoB29 was detected by western blot with anti-HA in the immunoprecipitate of cells transformed with pSLW1-B29 (B29, +) but not an empty vector control (-). Note that the polyubiquitinated material (last lane in the right figure) was only detected by western blot with anti-Myc in immunoprecipitate from cells expressing both ApoB29-HA and Ub-Myc. Lower molecular weight fragments most likely represent degradation intermediates and/or translation-arrested products.



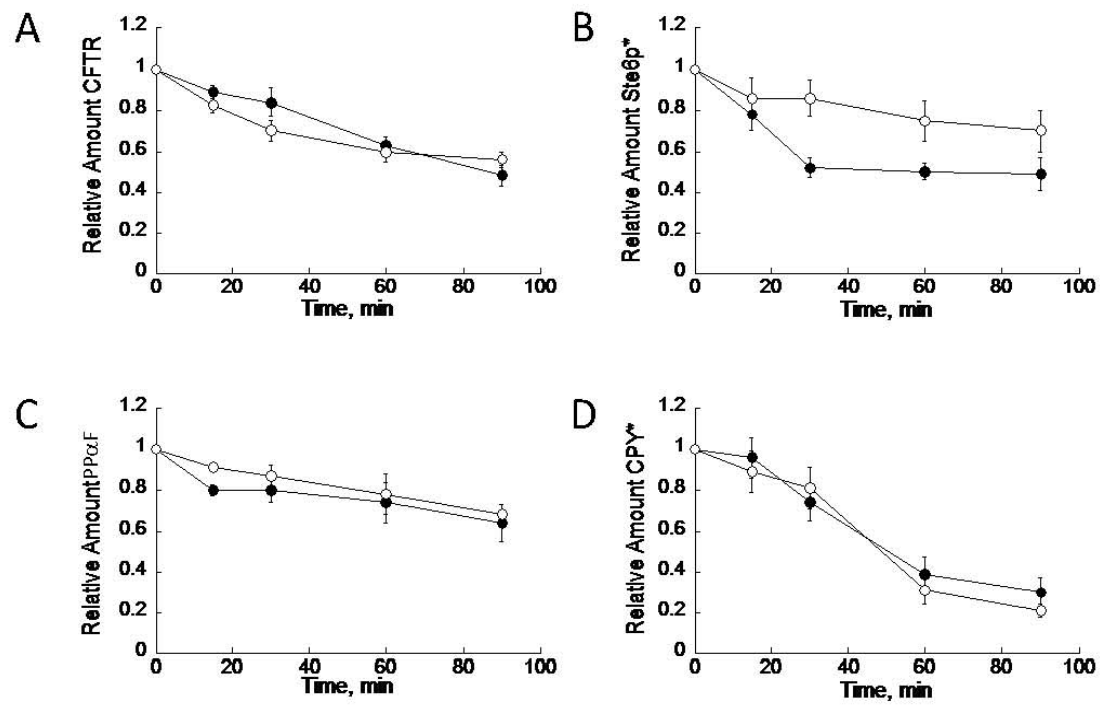
**Figure 23: ApoB29 Degradation Is Enhanced In *sse1Δ* Cells**

- A) A cycloheximide chase was performed in *SSE1* (●) and *sse1Δ* (*E0020*) cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA. L3 was used as a loading control. Data represent the means from 8 independent experiments  $\pm$  SE of the means. 60 min,  $p < 0.01$ ; 90 min,  $p < 0.004$
- B) A cycloheximide chase was performed in *SSB1SSB2* (●) and *ssb1Δssb2Δ* cells (○) transformed with pSLW1-B29. Data represent the means from 5 independent experiments  $\pm$  SE of the means.
- C) ApoB29 co-precipitates with Sse1p and Ssa1p, but not with Ssb1p. Extracts were prepared from cells transformed with a vector control or with pSLW1-B29 (B29) and were treated with anti-Sse1p, anti-Ssa1p, or anti-Ssb1p, and protein A sepharose. The proteins in the precipitates were resolved by SDS-PAGE and were immunoblotted with the indicated antisera.

(Goeckeler et al., 2002) was used to immunoprecipitate the chaperone. I found that ApoB29 only precipitated from strains that expressed Sse1p and ApoB29 (Figure 23C).

Because all of the Sse1p in the cell was reported to be in heterodimeric complexes with either Ssb1p or Ssa1p (the Hsp70 already shown to impact ApoB degradation) (Shaner et al., 2005), I wanted to determine if Ssb1p contributed to ApoB degradation. Surprisingly, the degradation of ApoB was unchanged in cells lacking Ssb1p and Ssb2p (Figure 23B). I wanted to ascertain if ApoB interacted with Ssb1p even though deletion of this Hsp70 did not impact the rate of ApoB degradation. ApoB29 failed to co-precipitate with Ssb1p, implying that the Sse1p-ApoB interaction occurs independently of this Hsp70 (Figure 23C). However, a putative Ssb1p-ApoB complex could easily disassociate, thus making it difficult to detect by immunoprecipitation. In contrast, when I immunoprecipitated Ssa1p, the Hsp70 that facilitates ApoB degradation in yeast (Gusarova et al., 2001), ApoB29 resided in the precipitate. Therefore, while both Sse1p and Ssa1p are proposed to be in a heterodimeric complex and I can detect the interaction of these chaperones with ApoB, they impact the ERAD of this substrate differently.

I next addressed whether the enhanced degradation of ApoB in the *sse1Δ* strain is substrate-specific. I found that there was no difference in the rate of degradation of CFTR, CPY\*, and *pac* in the *SSE1* and *sse1Δ* strains (Figure 24). In contrast, Ste6p\* stabilization was observed in the *sse1Δ* strain (Figure 24B). Others have reported that there is no difference in the rate of degradation of CPY\* lacking its signal sequence in *sse1Δ* and wildtype cells, but reduced degradation of VHL was noted in strains lacking Sse1p (McClellan et al., 2005; Park et al., 2007). Although these collective data indicate diverse effects on the turn-over of ERAD substrates in Sse1p-deficient strains, none of the substrates tested exhibited the enhanced



**Figure 24: Sse1p Differentially Impacts The ERAD Of Diverse Substrates**

A) A cycloheximide chase was performed in *SSE1* (●) or *sse1Δ* (*E0020*) cells (○) transformed with pRS426-CFTR-HA. CFTR was detected using anti-HA antibodies and Sec61p was used as a loading control. For all cycloheximide chase analysis, each time point was normalized to the Sec61p loading control and the relative amount of CFTR was calculated by dividing the values at each time by the value at  $t = 0$ . Data represent the means from 6 independent experiments  $\pm$  SE of the means. All experiments were performed at 30°C.

B) A cycloheximide chase was performed in *SSE1* (●) or *sse1Δ* (*E0020*) cells (○) transformed with pSM1082-Ste6p\*-HA. Ste6p\* was detected using anti-HA antibodies and Sec61p was used as a loading control, as above. Data represent the means from 6 independent experiments  $\pm$  SE of the means: 60 min,  $p < 0.006$ ; 90 min,  $p < 0.007$

C) A pulse chase was performed on  $^{35}\text{S}$  methionine labeled *SSE1* (●) or *sse1Δ* (*E0020*) cells (○) transformed with pSM36-pp $\alpha$ f $\Delta$ G-HA. P $\alpha$ f was immunoprecipitated from the cell extracts using anti-HA antibodies. Data represent the means from 4 independent experiments  $\pm$  SE of the means.

D) A pulse chase was performed on  $^{35}\text{S}$  methionine labeled *SSE1* (●) or *sse1Δ* (*E0020*) cells (○) transformed with pRS315-CPY\*-HA. CPY\* was immunoprecipitated from the cell extracts using anti-HA antibodies. Data represent the means from 4 independent experiments  $\pm$  SE of the means.

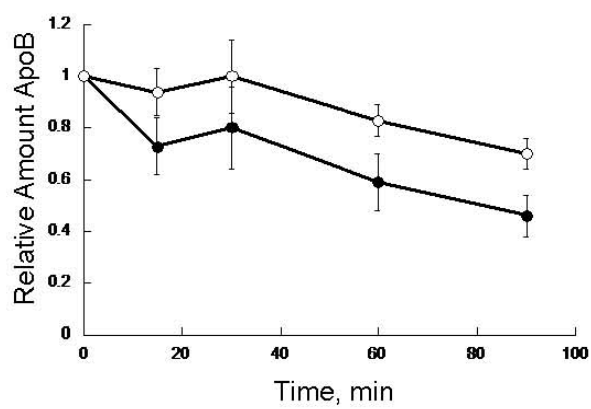
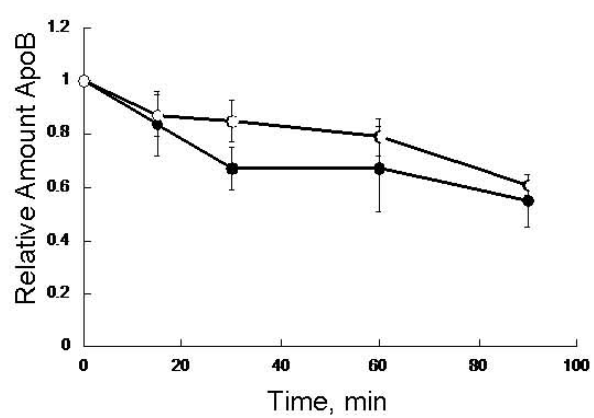
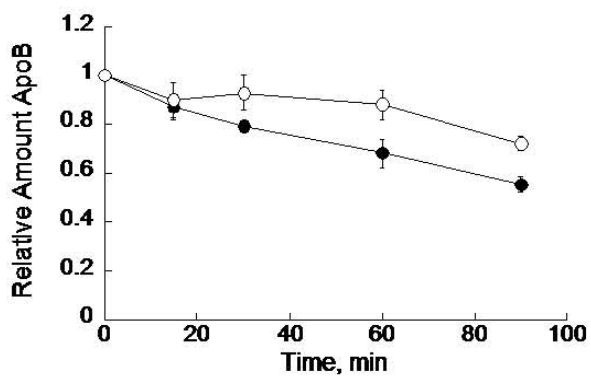
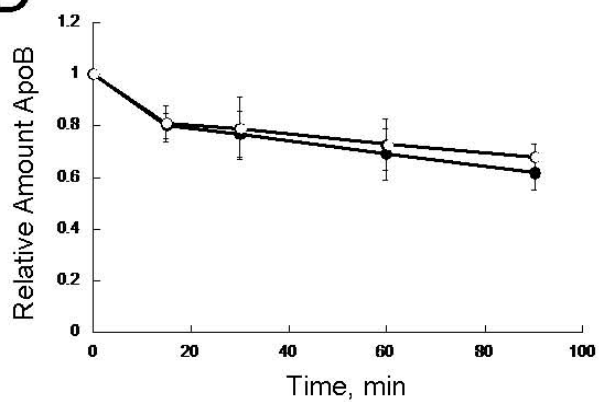


degradation I observed for ApoB29 in the *sse1Δ* strain. Overall, Sse1p appears to play a unique role as a stabilizing chaperone during the ERAD of ApoB.

#### **2.2.4 The Contribution Of Hsp70s and Hsp40s To ApoB Degradation**

The cytosolic Hsp40s, Ydj1p and Hlj1p, are functionally redundant, modulate the activity of Ssa1p, and are reported to genetically interact with Sse1p (Cyr, 1995; Cyr and Douglas, 1994; Cyr et al., 1992; Goeckeler et al., 2002; Huyer et al., 2004; Lu and Cyr, 1998; Tsai and Douglas, 1996; Youker et al., 2004). Because Sse1p is a pro-stabilization factor and Ssa1p is a pro-degradation factor for ApoB, I was interested to determine if the Hsp40s that interact with these chaperones also contribute to ApoB turnover. When examined by cycloheximide chase analysis, the degradation of ApoB was reduced in a yeast strain containing the temperature sensitive *ydj1-151* allele and that was deleted for *HLJ1*, suggesting that these Hsp40s may participate in the targeting of ApoB for degradation (data by J. McBride, S.L.H. and J.L.B, Figure 25A).

The ER lumenal Hsp70, BiP (Kar2p in yeast), has been reported to interact with ApoB and travel with ApoB from the ER to the Golgi in mammalian cells (Zhang and Herscovitz, 2003). In addition, a link between BiP and ApoB turnover has been established because ApoB turnover was enhanced in cells overexpressing BiP (Qiu et al., 2004). I examined the degradation of ApoB in yeast expressing a mutant allele of the Hsp70, *kar2-1*, that has been shown to compromise the ERAD of other substrates (Brodsky et al., 1999; Huyer et al., 2004; Kabani et al., 2003). ApoB was stabilized in the *kar2-1* strain compared to the wildtype strain suggesting that this ER lumenal Hsp70 is important for ApoB degradation (Figure 25C). I then examined the role of the ER lumenal Hsp40s reported to cooperate with Kar2p in refolding

**A****B****C****D**

**Figure 25: The Degradation Of ApoB In Hsp40, Hsp70, And NEF Mutant Yeast**

A) A cycloheximide chase was performed in *YDJ1HLJ1* (●) or *ydj1-151hlj1Δ* cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. For all cycloheximide chase analyses, each time point was normalized to the Sec61p loading control and the relative amount of ApoB was calculated by dividing the values at each time by the value at  $t = 0$ . Data represent the means from 7 independent experiments  $\pm$  SE of the means: 90 min,  $p < 0.02$

B) A cycloheximide chase was performed in *SCJ1JEM1* (●) or *scj1Δjem1Δ* cells (○) transformed with pSLW1-B29. ApoB29 was detected using anti-HA antibodies and Sec61p was used as a loading control. Data represent the means from 9 independent experiments  $\pm$  SE of the means.

C) A cycloheximide chase was performed in *KAR2* (●) or *kar2-1* cells (○) transformed with pSLW1-B29. Data represent the means from 5 independent experiments  $\pm$  SE of the means: 60 min,  $p < 0.03$ ; 90 min,  $p < 0.005$

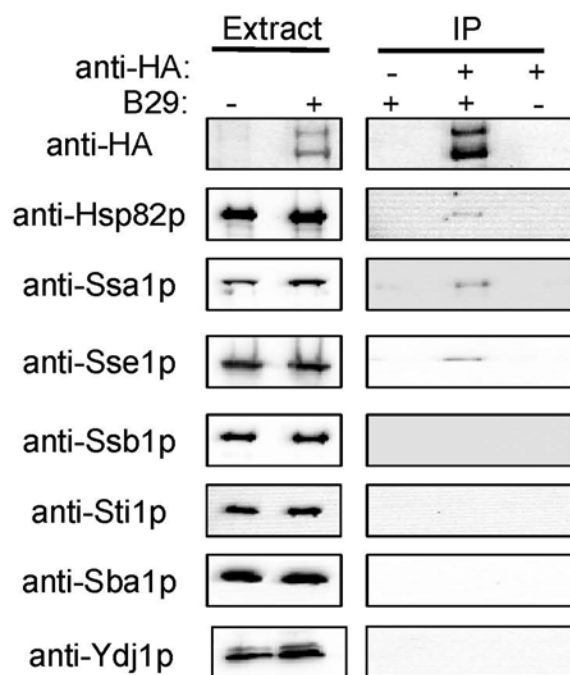
D) A cycloheximide chase was performed in *SNL1FES1* (●) or *snl1Δfes1Δ* cells (○) transformed with pSLW1-B29. Data represent the means from 9 independent experiments  $\pm$  SE of the means.

proteins and targeting proteins to ERAD, Scj1p and Jem1p, but I observed no difference in degradation between the wildtype cells and the *jem1Δscj1Δ* cells suggesting that while Kar2p contributes to ApoB degradation, the cognate Hsp40s are not pro-degradation factors for ApoB (data by J. McBride, S.L.H., and J.L.B. Figure 25B). Because Sse1p is also reported to function as a NEF for Hsp70, I examined the contribution of Snl1p and Fes1p to ApoB degradation. Yeast deleted for both of these NEFs exhibited no difference in ApoB degradation compared to the wildtype control (Figure 25D). Suggesting that it is not the reduction in Hsp70 nucleotide exchange factors alone that contributes to the enhanced degradation of ApoB in cells lacking Sse1p.

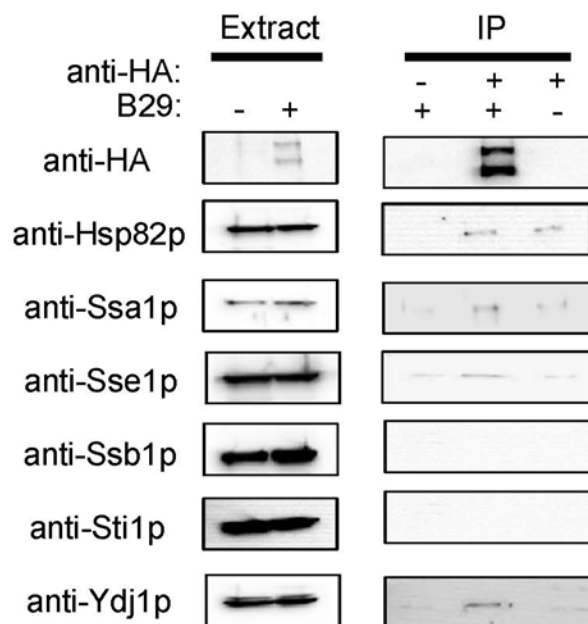
#### **2.2.5 ApoB Does Not Interact With All Of The Components Of The Hsp90 Complex**

All of the data present above indicates that ApoB degradation depends on several factors in the Hsp90 complex: Hsp82p, Ssa1p, Sse1p, and Ydj1p. Does ApoB interact with all of the components of the Hsp90 complex? To answer this question, the protein was again immunoprecipitated using non-denaturing conditions and Hsp82p, Ssa1p and Sse1p were detected in the precipitate by western blot. However, I failed to observe Ssb1p, Sti1p or Sba1p co-precipitating with ApoB (Figure 26A). Ydj1p co-precipitated with ApoB under conditions utilizing wash buffers containing varying amounts of Tween20, however washes with high salt disrupted the interaction (Figure 26A,B). It should be noted that Sti1p, Sba1p, and Ssb1p were not detected interacting with ApoB under any conditions tested. Therefore, chaperones that impact ApoB ERAD (i.e., Sse1p, Ssa1p, Hsp82p, Ydj1p) associate with ApoB, whereas factors

A



B



**Figure 26: ApoB29 Co-Precipitates With Hsp82p, Ssa1p, Ydj1p And Sse1p, But Not With Ssb1p, Sti1p, Or Sba1p.**

A) Extracts were prepared from cells transformed with a vector control or with pSLW1-B29 (B29) and were treated with anti-HA resin or an unconjugated sepharose resin control. The resin was washed with buffers containing 150mM and 300mM NaCl. The proteins in the precipitates were resolved by SDS-PAGE and were immunoblotted with the indicated antisera.

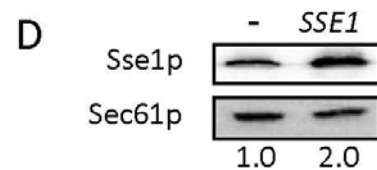
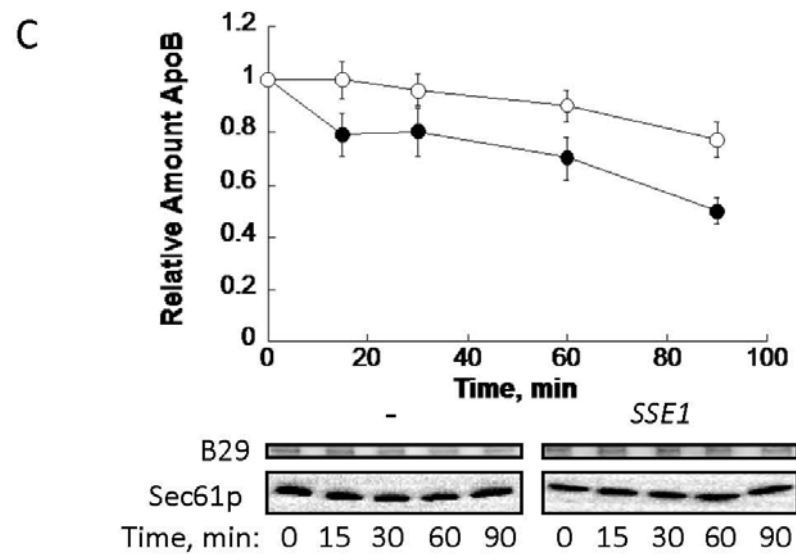
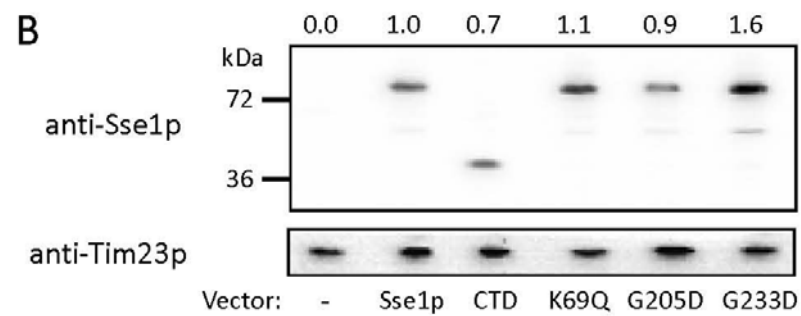
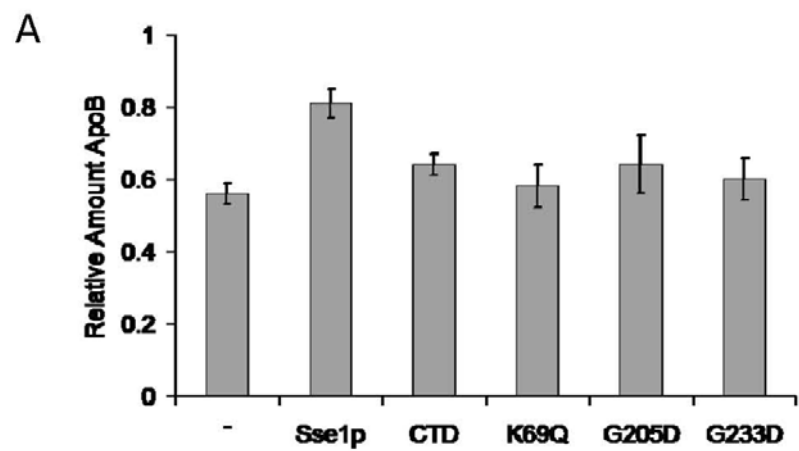
B) Immunoprecipitations were performed as in described in part A, however the wash buffers utilized contained sequentially 0.5% Tween20 and 1% Tween20. Note that Ydj1p only co-precipitates with ApoB in the detergent washes but not after the high salt washes. In addition, more Ssa1p and Hsp82p are found non-specifically interacting with the HA resin after the Tween20 washes.

that have no effect on the degradation of ApoB (see below, section 3.2.3) not detected with the lipoprotein (i.e., Ssb1p, Stilp, Sba1p).

## **2.2.6 The ATPase Activity Of Hsp110 Is Required For The Stabilization Of ApoB**

Unlike the Hsp70 chaperones, Sse1p is a “holdase” that retains proteins in solution but is unable to catalyze folding, and the N-terminal ATP-binding domain is dispensable for holdase activity (Goeckeler et al., 2002). However, the ability of Sse1p to support viability in some strain backgrounds may require ATP-binding—but not ATPase—activity (Shaner et al., 2004). To determine which features of Sse1p help stabilize ApoB, I assessed ApoB29 degradation in an *sse1Δ* strain transformed with Sse1p expression constructs that encode wildtype or mutant forms of the protein (Shaner et al., 2004). I first observed that cells expressing full-length Sse1p (from the introduced plasmid) stabilized ApoB29 compared to cells transformed with the vector control (Figure 27A), suggesting that modulating Hsp110 levels can affect ApoB turnover. However, cells expressing a K69Q mutant (that binds ATP but is unable to hydrolyze nucleotide), Sse1p-G205D (that is unable to bind ATP), Sse1p-G233D (that cannot bind Ssa1p and lacks ATPase activity), and the C-terminal peptide-binding domain of Sse1p that maintains some holdase activity (CTD; (Goeckeler et al., 2002)) did not significantly stabilize ApoB, even though the proteins were expressed to similar or even higher levels than the wildtype control (Figures 27A,B).

I next asked if increased amounts of Sse1p would also stabilize ApoB29 in yeast. Sse1p was over-expressed from an introduced plasmid about 2-fold and enhanced stabilization of ApoB29 was noted when compared to control cells (Figure 27C,D). Interestingly, these conditions were unable to lead to detectable ApoB secretion (data not shown). This phenomenon





**Figure 27 The Sse1p ATP-Binding Domain Is Required For ApoB29 Stabilization**

- A) ApoB29 degradation was assessed by cycloheximide chase analysis in *sse1Δ (W303)* cells transformed with pSLW1-B29 and the indicated Sse1p expression constructs (see Table 6). Data correspond to the means of the 90 min time point from 6 independent experiments  $\pm$  SE of the means:  $p < 0.004$  for cells transformed with the control vector versus cells transformed with the wild type expression vector.
- B) Relative levels of Sse1p expression in each strain were examined and compared to the amount of full length Sse1p in the wildtype control. Values above the blot correspond to the means for 4 independent blots. The mitochondrial protein, Tim23p, was used as a loading control.
- C) ApoB29 degradation was assessed by cycloheximide chase analysis in *SSE1 (E0020)* cells transformed with pSLW1-B29 and the p414TEF-*SSE1* expression vector ( $\circ$ ) or an empty vector control ( $\bullet$ ). Data represent the means from 6 independent experiments  $\pm$  SE of the means. 60 min,  $p < 0.02$ ; 90 min,  $p < 0.005$
- D) Sse1p steady state levels increased 2 fold in the cells transformed with p414TEF-*SSE1* compared to the empty vector control. Sse1p was detected using anti-Sse1p. Sec61p was used as a loading control. Data represent the means from 3 independent experiments.

was also evident when ApoB100 was examined in HepG2 cells that had been treated with proteasome inhibitors, i.e., an increase in intracellular ApoB did not lead to an increase in secreted material (data not shown) (Mitchell et al., 1998). In any event, these data do indicate that a modest increase in Sse1p protects ApoB from ERAD.

### **2.3 GENERAL SUMMARY OF THE IN VIVO YEAST SYSTEM RESULTS**

In this chapter I have described a new yeast expression system for ApoB in which the protein is targeted to the ER and requires components of the UPP for degradation. However, unlike in mammalian cells, ApoB does not appear to be secreted from yeast and oxidative stress does not appear to enhance ApoB degradation. Utilizing this system I obtained data that identified the cytosolic Hsp110, Sse1p, as the first pro-stabilization chaperone identified for ApoB. This stabilization activity is dependent on the ATPase activity of Sse1p and the stabilization of ApoB can be enhanced by increasing the amount of Sse1p in the cell. Although Sse1p is found in complexes with both Hsp70s, Ssa1p and Ssb1p, only Ssa1p immunoprecipitates with and impacts the degradation of ApoB. Interestingly, even though Sse1p and Ssa1p are found in a complex together, they have opposing roles in ApoB degradation. The cytosolic Hsp40s, Ydj1p and Hlj1p, that interact with Ssa1p are also pro-degradation factors for ApoB. In contrast, while ApoB requires the ER luminal Hsp70, Kar2p (the yeast homolog of BiP), for degradation, the cognate Hsp40s, Scj1p and Jem1p, do not contribute to this process.

### **3.0 EXAMINATION OF APOLIPOPROTEIN B ERAD USING AN IN VITRO SYSTEM AND CONFIRMATION OF YEAST RESULTS IN A MAMMALIAN CELL CULTURE SYSTEM**

Previous work by the Fisher lab established an in vitro degradation assay for the human ApoB48 isoform (Gusarova et al., 2001). In this assay, ApoB48 is translated in the presence of dog pancreas microsomes and <sup>35</sup>S labeled methionine, and after re-isolation of the ApoB-containing vesicles, the degradation of the radiolabeled substrate can be measured in the presence of either mammalian or yeast cell lysates. ApoB48, which is ~48% of the size of full-length ApoB (260 kDa), is expressed endogenously and secreted from rodent hepatic and rodent and human intestinal cells, and undergoes MTP and lipid-dependent maturation (Wu and Windmueller, 1981). In addition, polyubiquitinated ApoB can be detected in the translation reaction and the proteasome dependent degradation of ApoB can be achieved with either mammalian cell extracts or yeast cytosol. ApoB was found to require both Hsp70 and Hsp90 for degradation in yeast cytosol and these results were confirmed in mammalian cell culture (Gusarova et al., 2001; Zhou et al., 1995).

This section contains experiments in which I utilized this in vitro degradation system to confirm and extend the results I obtained with the in vivo system (Chapter 2.0). First, I found that ApoB requires the Cdc48p complex for degradation in both systems. Second, in a modification to the previous system which utilized rabbit reticulocyte lysate with exogenously

added HA-tagged ubiquitin to detect polyubiquitinated ApoB (Gusarova et al., 2001), I was able to immunoprecipitate polyubiquitinated ApoB from reactions containing cytosol from yeast overexpressing a HA-tagged ubiquitin construct. Third, I recapitulated the enhanced degradation of ApoB in cytosol from yeast cells lacking Sse1p. The enhanced degradation of ApoB was not the result of increased proteasome levels in the cytosol and could be abrogated by the addition of purified Sse1p, further indicating that Sse1p is a pro-stabilization chaperone for ApoB. In addition, ApoB degradation was similar in cytosol from *ssb1 $\Delta$ ssb2 $\Delta$*  cells and the corresponding wildtype strain, again confirming results I obtained with the in vivo system. Fourth, as the in vitro system utilizes a larger isoform of ApoB than the in vivo system (ApoB48 vs ApoB29), I constructed an ApoB29 expression plasmid for use in the in vitro system. ApoB29 exhibited the same enhanced degradation as ApoB48 in the presence of *sse1 $\Delta$*  cytosol, suggesting that these two isoforms are processed by ERAD in a similar manner. Fifth, I examined the degradation of ApoB in yeast cytosol prepared from *stil $\Delta$ sba1 $\Delta$*  yeast and observed no effect on ApoB turnover. And finally, to confirm that Hsp110 is involved in stabilizing ApoB in a mammalian system, I conducted pulse chase experiments using a rat hepatoma cell line. When Hsp110 was overexpressed, more ApoB was observed intracellularly at 15 min and secreted into the media at 60 min. Similar results were obtained when the cells were treated with an MTP inhibitor (thus favoring ApoB ERAD (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Fisher et al., 1997; Liao et al., 1998; Sakata and Dixon, 1999; Yeung et al., 1996)) and oleic acid (OA, a fatty acid that stimulates ApoB secretion (Homan et al., 1991; Mitchell et al., 1998; Pan et al., 2002)). Therefore, Hsp110 is a stabilization factor for ApoB that promotes ApoB secretion from mammalian cells and is a novel drug target for patients with high cholesterol.

### **3.1 METHODS AND MATERIALS**

#### **3.1.1 In Vitro ApoB Degradation Assay**

ApoB48 degradation was assessed in vitro as previously described (Gusarova et al., 2001), except that time points were taken at 5, 15 and 30 min. Yeast strains utilized for cytosol preparations are listed in table 5. The cytosol used in the reactions was prepared as previously described (McCracken and Brodsky, 1996), and was diluted before use to a final concentration of 5-10 $\mu$ g/ $\mu$ l and pre-incubated with either 250 $\mu$ M MG132 (Peptides International) or an equivalent volume of DMSO for 15 min on ice. The reactions were quenched by adding an equal volume of 125mM Tris, pH 6.8, 4% SDS, 6M urea, 1mM EDTA, 10mM DTT, 250mM  $\beta$ -mercaptoethanol, 20% glycerol, 0.05% bromophenol blue, and the samples were heated at 96°C for 4 min prior to SDS-PAGE. Phosphorimager data were analyzed using Image Gauge software (Fuji Film Science Lab).

To examine the effect of purified Sse1p on ApoB stabilization, hexahistidine-tagged Sse1p was purified as previously described (Goeckeler et al., 2002), dialyzed into 1X PH buffer (20mM HEPES, pH7.4, 110mM KCl, 5mM MgCl<sub>2</sub>), and added into the cytosol at a final concentration of 3% of the total protein. The reaction was incubated on ice for 15 min before the degradation reaction commenced. In addition, Hsp82p was purified as previously described and dialyzed into 1X PH buffer (Youker et al., 2004). The activity of Hsp82p was confirmed by a previously described NBD1 aggregation assay (Strickland et al., 1997; Youker et al., 2004) before it was utilized in vitro to confirm that the chaperone contributed to ApoB degradation. Hsp82p was added in at a final concentration of 0.2 $\mu$ g/ $\mu$ L into the cytosol and incubated for 15 min before the degradation reaction was started.

To determine if the LIC peptide (LICGFRVVLMYRF) (synthesized by the University of Pittsburgh Peptide Synthesis Facility) which has been shown to bind Sse1p and inhibit its ATPase activity (J. Goeckeler and J.L.B. unpublished data) affected ApoB48 degradation in vitro, the peptide was dissolved in 1X PH buffer. LIC peptide was added to the cytosol to a final concentration of 50 $\mu$ M and 150 $\mu$ M and following a 15min pre-incubation on ice the radiolabeled ApoB was added to the mixture and the reaction commenced at 37°C for 30min. An equivalent volume of dimethylformamide (DMF) was added to the control reactions as this was the solvent utilized to dissolve the peptide.

The majority of in vitro degradation reactions were performed using the SP6-ApoB48 expression vector. However an additional ApoB29 expression vector was constructed using a site directed mutagenesis kit (Stratagene) with the primers (Forward 5' TGG TCC GCC TCC TAC AGT TGA TGA AAC ACC AGC ACA GAC C 3' and Reverse 5' GGT CTG TGC TGG TGT TTC ATC AAC TGT AGG AGG CGG ACC A 3') to insert tandem stop codons into the ApoB48 sequence to produce an SP6 expression construct that encoded ApoB29. The integrity of the plasmid was confirmed by sequence analysis.

### **3.1.2 Detecting Polyubiquitinated ApoB In Vitro**

The following was a modified protocol that was previously used by the Fisher lab (Gusarova et al., 2001). Yeast cytosol was prepared as previously described (McCracken and Brodsky, 1996) from cells transformed with a vector control or pSM990 (Copper inducible HA tagged ubiquitin construct (Hochstrasser et al., 1991)). Before harvesting, cells were treated for 1 hour with 100 $\mu$ M CuSO<sub>4</sub> and clarified cytosol was prepared (see section 3.1.1). S<sup>35</sup> labeled ApoB48 associated with canine pancreatic microsomes were isolated and reactions were set up as

described above. The reactions were incubated at 37°C or 30°C for 1 hour. The reactions were then diluted five-fold in 150mM Roche buffer with 10mM NEM, heated at 37°C for 3min and centrifuged at 10 000 rpm in a micro-centrifuge for 3min to pellet the microsomal membranes. The solubilized ApoB in the supernatant was immunoprecipitated with anti-HA resin (see section 2.1.4). Immunoprecipitated proteins were resolved by SDS-PAGE and the phosphorimager data were analyzed using Image Gauge software (Fuji Film Science Lab).

### **3.1.3 Immunoprecipitations From The In Vitro System**

ApoB48-chaperone complexes were immunoprecipitated from in vitro degradation reactions that were set-up as described above (section 3.1.1). The reactions were diluted five-fold in 1X PH buffer with 1% Triton X 100 and 20mM NaMoO<sub>4</sub>. The reactions were heated at 37°C for 3min and centrifuged at 10 000 rpm in a micro-centrifuge for 3min to pellet the microsomal membranes. The solubilized ApoB in the supernatant was incubated overnight at 4°C with the appropriate antibody (See Table 5) and protein A sepharose (GE Healthcare). The reactions were washed three times with the 1X PH buffer containing 1% Triton. Immunoprecipitated proteins were resolved by SDS-PAGE and the phosphorimager data were analyzed using Image Gauge software (Fuji Film Science Lab).

### **3.1.4 Proteasome Activity Assay**

The proteasome activity assay I utilized to confirm that the enhanced ApoB degradation in *sse1Δ* cytosol was not due to enhanced proteasome activity was conducted as previously described

(Glickman et al., 1998a; Scott et al., 2007). The immunoblot protocol and antibodies used to examine proteasome levels were also described (Scott et al., 2007).

### **3.1.5 Hsp110 Overexpression In Rat Hepatoma Cells**

Rat hepatoma McA-RH7777 cells were cultured and transfected with either the pcDNA3.1 vector (control) or the pcDNA3.1-Hsp110 expression vector (provided by Dr. J. Subjeck, Roswell Park Cancer Institute), and 48h after transfection a pulse-chase analysis was performed as previously described (Gusarova et al., 2001). Experiments to which the OA or the MTP inhibitor (BMS-200150) (Jamil, 1996) was added (at a final concentration of 0.625 $\mu$ M and 0.1nM respectively) measured the amount of ApoB-precipitable material from vector vs. Hsp110-overexpressing cells after a 15min pulse and 60min chase. Percent ApoB recovery was determined by dividing the ApoB signal normalized to albumin levels in the media at 60min by the ApoB signal normalized to albumin levels in the cell extracts at 15min.

## **3.2 RESULTS**

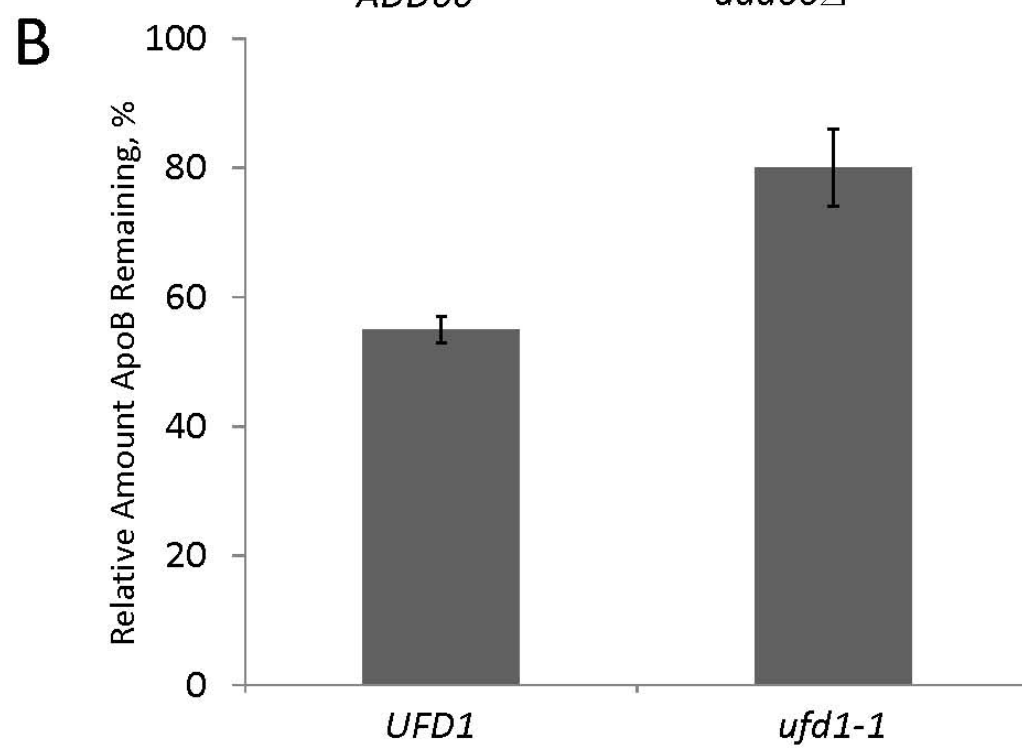
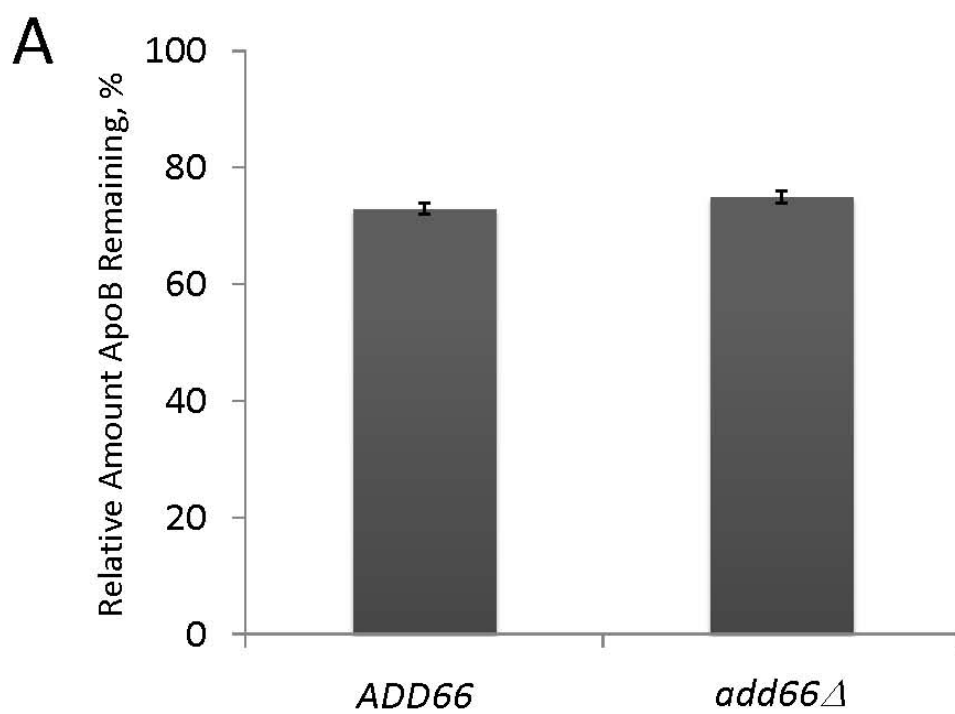
### **3.2.1 ApoB Is Degraded By The UPP In Vitro**

ApoB has previously been shown to be degraded in a proteasome dependent manner in yeast cytosol (Gusarova et al., 2001). In concordance with this previous result, I observed that the degradation of ApoB in yeast cytosol was abrogated in the presence of the proteasomal inhibitor MG132 (For an example see Figure 30B). Interestingly, when I examined the degradation of ApoB in cytosol from a strain lacking, Add66p, a proteasome assembly factor (Scott et al., 2007), I observed no effect on ApoB turnover. Add66p was originally identified as a factor that



impacted the degradation of the mutant serpin, A1PiZ, in yeast (Palmer et al., 2003; Scott et al., 2007). Therefore, even though this factor is important for another ERAD substrate, the delay in proteasomal assembly that occurs in the absence of Add66p had no effect on ApoB degradation, suggesting that the proteasome is not the rate limiting step in ApoB degradation (Figure 28A). In contrast, ApoB was stabilized in cytosol from *ufd1-1* cells expressing a mutant component of the Cdc48p complex, a group of proteins shown to be important for recognizing polyubiquitinated substrates and targeting them to the proteasome (data by D. Schleicher, S.L.H. and J.L.B) (Figure 28B).

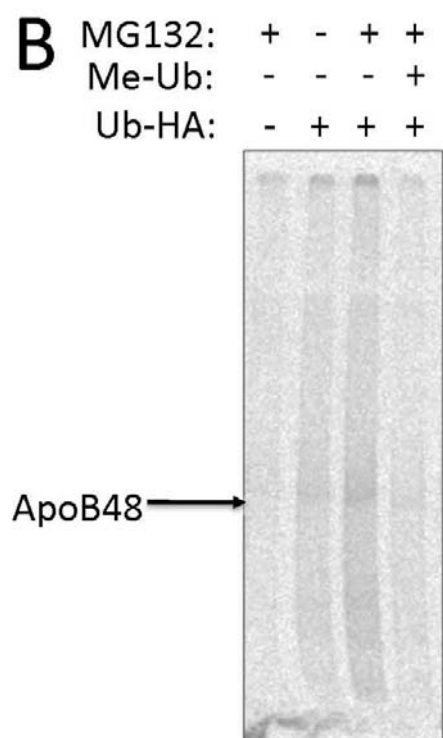
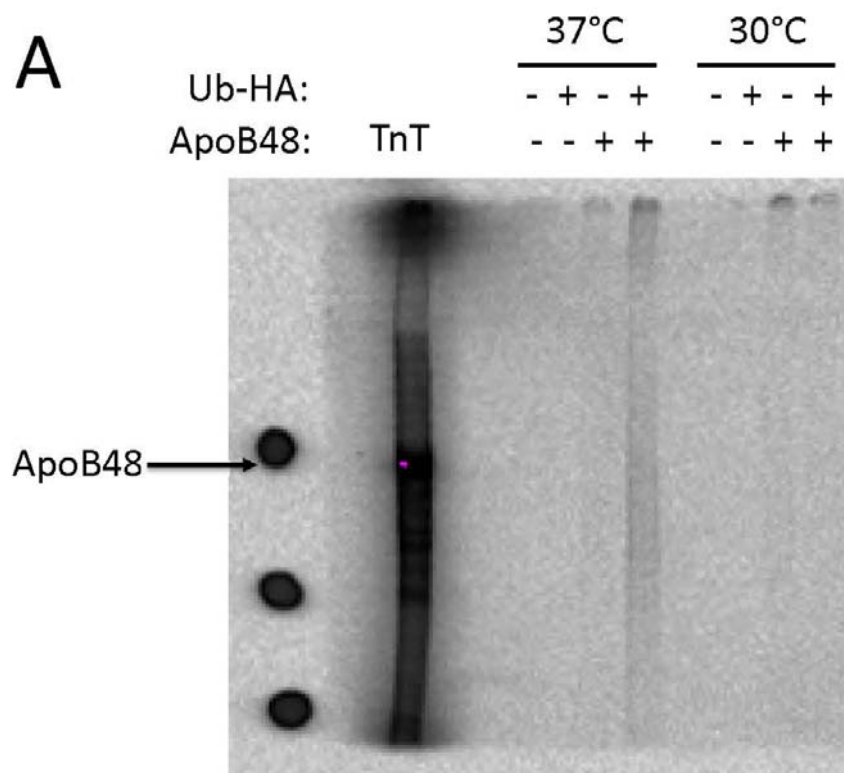
Previously, the Fisher lab had reported the ability to immunoprecipitate polyubiquitinated ApoB from the in vitro transcription/translation reaction when the rabbit reticulocyte lysate was supplemented with purified HA tagged ubiquitin (Gusarova et al., 2001). In order to determine if ApoB is further polyubiquitinated upon the addition of yeast cytosol, I incubated ApoB-canine pancreatic microsomes with yeast cytosol from cells overexpressing an HA-tagged ubiquitin construct. In a similar manner, I was able to immunoprecipitate radiolabeled ApoB in preliminary experiments in the presence of both anti-HA and the HA tagged ubiquitin as indicated by the ‘smear’ of radiolabeled material in the precipitate (Figure 29A). Importantly, the amount of polyubiquitinated ApoB isolated was modestly decreased when methyl-Ub, a ubiquitin moiety that blocks additional ubiquitin conjugation (Hershko and Heller, 1985), was added to the reaction (Figure 29B). The ideal temperature for the polyubiquitination reaction is 37°C, which is the temperature utilized for the ApoB48 in vitro degradation reactions, not 30°C which has been previously utilized to examine Ste6p\* polyubiquitination in vitro (Nakatsukasa et al., 2008).



**Figure 28: ApoB Requires A Component Of The Cdc48p Complex For Degradation In Vitro**

A) ApoB48 degradation was assessed in vitro at 37°C for 30 min using cytosol at a final concentration of 5µg/µL from *ADD66* or *add66Δ* yeast. Add66p is a proteasome assembly factor in yeast and cytosol from cells lacking the protein exhibit reduced chymotrypsin-like protease activity due to a proteasome assembly defect (Scott et al., 2007). The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation (i.e., degradation in the presence of MG132 would be denoted as 100%). Data represent the means from 7 independent experiments  $\pm$  SE of the means.

B) ApoB48 degradation was assessed in vitro at 37°C for 30 min using cytosol at a final concentration of 10µg/µL from *UFD1* or *ufd1-1* mutant yeast. Ufd1p is a protein component of the Cdc48p complex and the *ufd1-1* allele has been previously shown to exhibit an ERAD defect for some protein substrates (Gnann et al., 2004). The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 15 independent experiments  $\pm$  SE of the means ( $p < 0.0001$ ).



### **Figure 29: ApoB Appears To Be Polyubiquitinated In Vitro In Yeast Cytosol**

A) ApoB48 associated with microsomes prepared as described in (Nakatsukasa et al., 2008) was incubated in cytosol at a final concentration of 10µg/µl from yeast cells expressing HA-tagged ubiquitin (Ub-HA, +) or transformed with an empty vector (-). After a 30min reaction at 30°C or 37°C, polyubiquitinated ApoB was immunoprecipitated with anti-HA resin. The transcription and translation reaction was included on the gel (TnT) in order to visualize full length ApoB48. The TnT lane is overexposed compared to the ApoB signal in the immunoprecipitation reaction, indicating that a small pool of ApoB is further polyubiquitinated upon exposure to yeast cytosol.

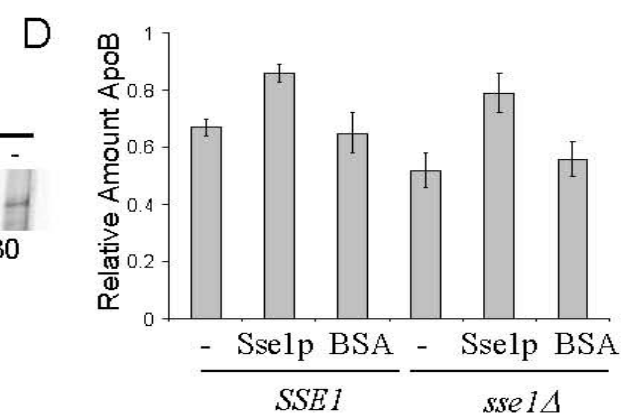
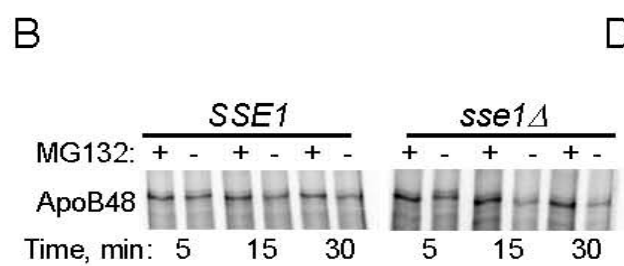
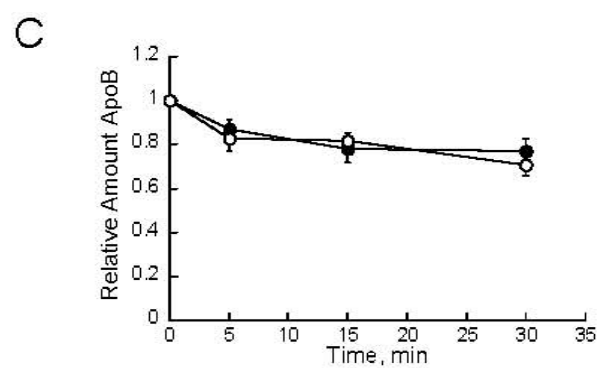
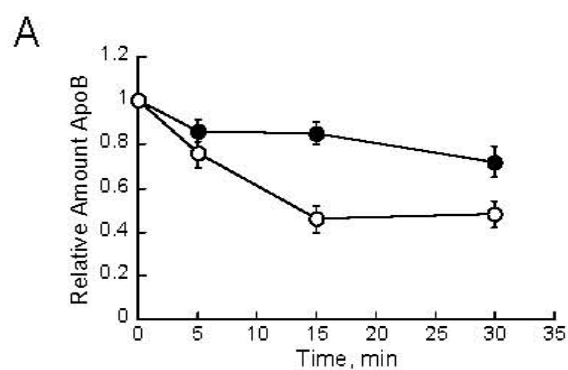
B) The ubiquitination reaction for ApoB in yeast cytosol was conducted as above but in the presence of DMSO or MG132 (the proteasomal inhibitor). The cytosol from cells transformed with an empty vector (-) and thus not expressing Ub-HA and the addition of 3µg of methyl-ubiquitin (Me-Ub) served as negative controls for the polyubiquitination reaction. Note that there is a slight increase in the amount of polyubiquitinated ApoB precipitated in the reaction containing MG132 (lane 3).

It is vital to note that the experiments in parts A and B are preliminary and further testing of conditions to optimize ApoB polyubiquitination in yeast cytosol needs to be performed. For example, the signal-to-noise ratio needs to be improved and the presence of low molecular weight ubiquitin species that may arise from translation or degradation intermediates need to be examined further.

### 3.2.2 Hsp110 Stabilizes ApoB In Vitro

To confirm and extend the results obtained in vivo (Chapter 2.0), I utilized cytosols prepared from *sse1Δ* yeast and an isogenic wild type strain in the previously established in vitro ERAD assay (Gusarova et al., 2001). As published previously (Gusarova et al., 2001), ~70% of the ApoB remained after 30 min in a reaction supplemented with cytosol from a wild type yeast strain. In contrast, when ApoB degradation was examined in the presence of cytosol from the *sse1Δ* strain, only ~50% of the translated ApoB remained following a 30min incubation (Figure 30A,B).

Sse1p is reported to interact with and modulate the ATPase activity of two cytosolic Hsp70s in yeast, Ssa1p and Ssb1p (Dragovic et al., 2006a; Raviol et al., 2006b; Yam et al., 2005). Even though Ssa1p facilitates ApoB degradation in vitro (Gusarova et al., 2001), Ssb1p, an Hsp70 in the RAC, did not contribute to ApoB degradation in vivo ( see above Figure 23B). In order to recapitulate these results in vitro, cytosols were prepared from a strain deleted for *SSB1* and the *SSB2* homologue and from an isogenic wildtype strain. Next, ApoB degradation was assessed in vitro (Figure 30C). In contrast to the pro-degradative effect of Ssa1p on ApoB and the stabilizing effect of Sse1p on ApoB, I again found that the levels of ApoB were unaltered when the Ssb chaperones were absent. It should be noted that the extent of degradation observed was lower than in Figure 30A, which is consistent with the previously observed difference in ApoB ERAD when different yeast strain backgrounds were utilized (Gusarova et al., 2001). Nevertheless, the in vitro and in vivo data suggest that individual Hsp70s and Hsp70 facilitators



**Figure 30: Sse1p Contributes To ApoB48 Stabilization In Vitro**

A) ApoB48 degradation was assessed in vitro at 37°C using cytosol (5µg/µL) from *SSE1* (●) or *sse1Δ* (*JG014a*) (○) yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 6 independent experiments  $\pm$  SE of the means: 15 min;  $p < 0.0002$ , 30 min;  $p < 0.0005$

B) Phosphorimage of  $^{35}\text{S}$  labeled ApoB48 during a representative degradation assay in cytosol from *SSE1* or *sse1Δ* (*JG014a*) yeast. Reactions were treated with the proteasome inhibitor MG132 (250µM) or DMSO (-) as indicated.

C) ApoB48 degradation is similar in cytosol from *ssb1Δssb2Δ* cells and the isogenic wildtype cells. ApoB48 degradation was assessed in vitro at 37°C using cytosol (5µg/µL) from *SSB1SSB2* (●) or *ssb1Δssb2Δ* (○) yeast, and quantified as above. Data represent the means from 6 independent experiments  $\pm$  SE of the means.

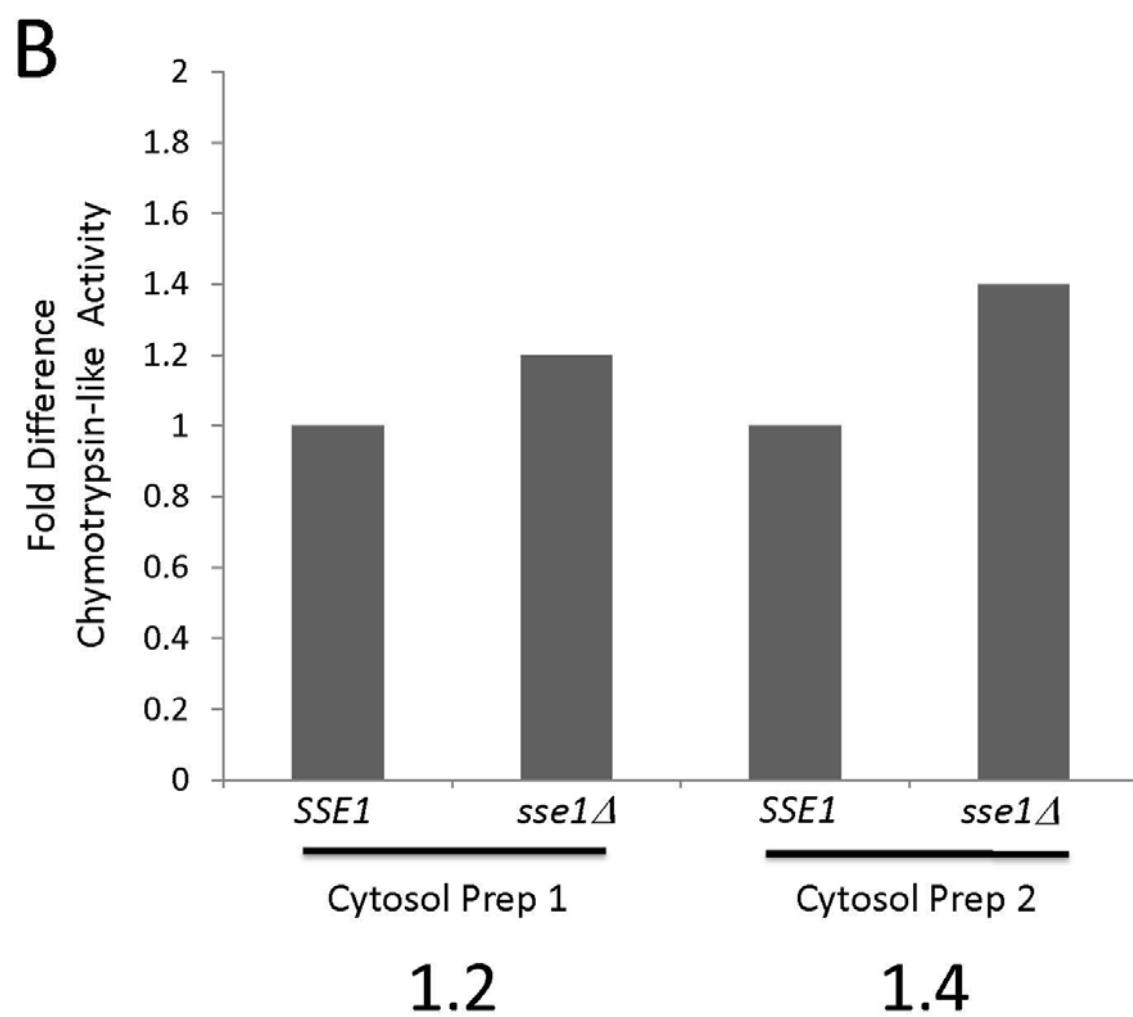
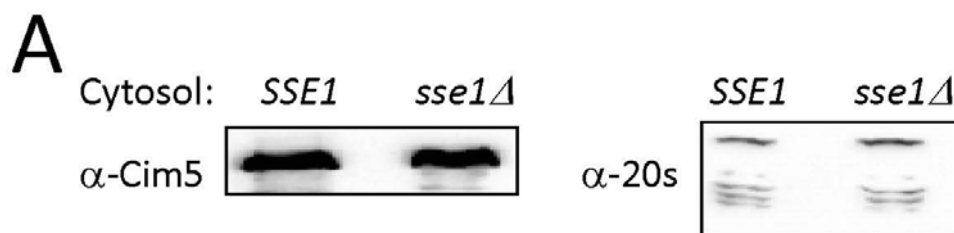
D) Degradation reactions containing the indicated source of cytosol at a final concentration of 5µg/µl were supplemented with Sse1p at a final concentration of 3% of the total protein or an equal amount of bovine serum albumin (BSA). Reactions were incubated at 37°C for 15 min. Data represent the means of 3 to 9 experiments  $\pm$  SE of the mean. The activity of purified Sse1p was confirmed in steady state ATPase assays: 0.5 nmole ATP hydrolyzed  $\text{min}^{-1} \text{mg protein}^{-1}$ . *SSE1* cytosol +/- purified Sse1p,  $p < 0.02$ ; *sse1Δ* (*JG014a*) cytosol +/- purified Sse1p,  $p < 0.002$



can exert unique effects during the biogenesis of a given polypeptide, and point to the complexity with which these chaperones and co-chaperones act.

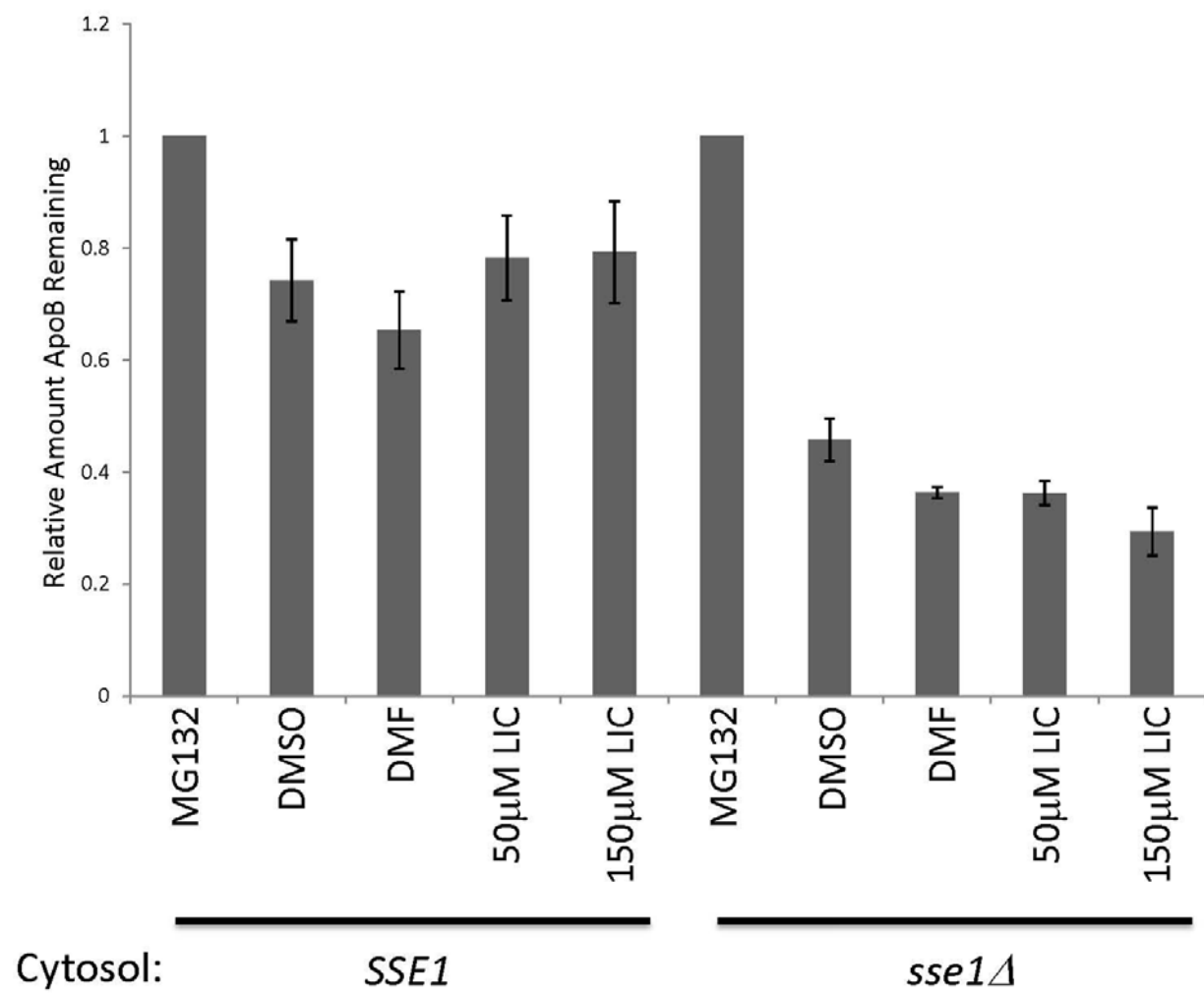
To determine if the enhanced degradation of ApoB in cytosols lacking Sse1p was specific and not the result of secondary consequences, purified Sse1p was supplemented into the degradation reactions. As shown in Figure 30D, Sse1p stabilized ApoB regardless of whether it was added to cytosols prepared from *sse1Δ* cells or the isogenic wildtype strain. The addition of an irrelevant protein, bovine serum albumin (BSA), had no impact on ApoB degradation. Furthermore, when I examined the relative proteasome levels in cytosol from wildtype and *sse1Δ* cells by quantitative western blot analysis I observed no significant change in relative proteasome levels between the two extracts (Figure 31A). To determine if the activity of the proteasome in the cytosol preparations was significantly different I evaluated the chymotrypsin-like activity of the extracts. I observed a slight enhancement of chymotrypsin-like activity in the *sse1Δ* cytosol compared to the *SSE1* cytosol (Figure 31B). However, increased proteasome activity alone cannot account for the enhanced degradation of ApoB in *sse1Δ* cytosol as demonstrated by the rescue experiments with purified Sse1p. In addition, I have previously shown that a cytosol from a strain with reduced chymotrypsin like activity (*add66Δ*) (Scott et al., 2007) exhibited no ApoB degradation defect (see above, Figure 28A).

Finally, Jen Goeckeler in our lab has identified a peptide (LIC) that exhibits a significantly stronger binding affinity for Sse1p than Ssa1p in vitro. However, in her experiments she observed inhibition of the ATPase activity of both Sse1p and Ssa1p in the presence of the LIC peptide. I tested if addition of LIC to the degradation reaction specifically altered the activity of Sse1p resulting in a change in ApoB degradation rates. However, similar



**Figure 31: Proteasome Levels And Activity Are Similar in *SSE1* and *sse1Δ* cytosol.**

A) Approximately 15μg of cytosol from *SSE1* and *sse1Δ* cells were probed for both Cim5p, a component of the 19S cap, and the 20S proteasomal subunits. There is no significant difference in the levels of these proteasome subunits as determined by quantification with the Kodak 1D v3.6 software (Data not shown). B) The chymotrypsin-like activity of two different cytosol preparations from *SSE1* and *sse1Δ* cells were examined and a ~27% increase in activity was observed in the *sse1Δ* cytosols. Chymotrypsin-like activity is the predominant proteolytic activity of the 26S proteasome.



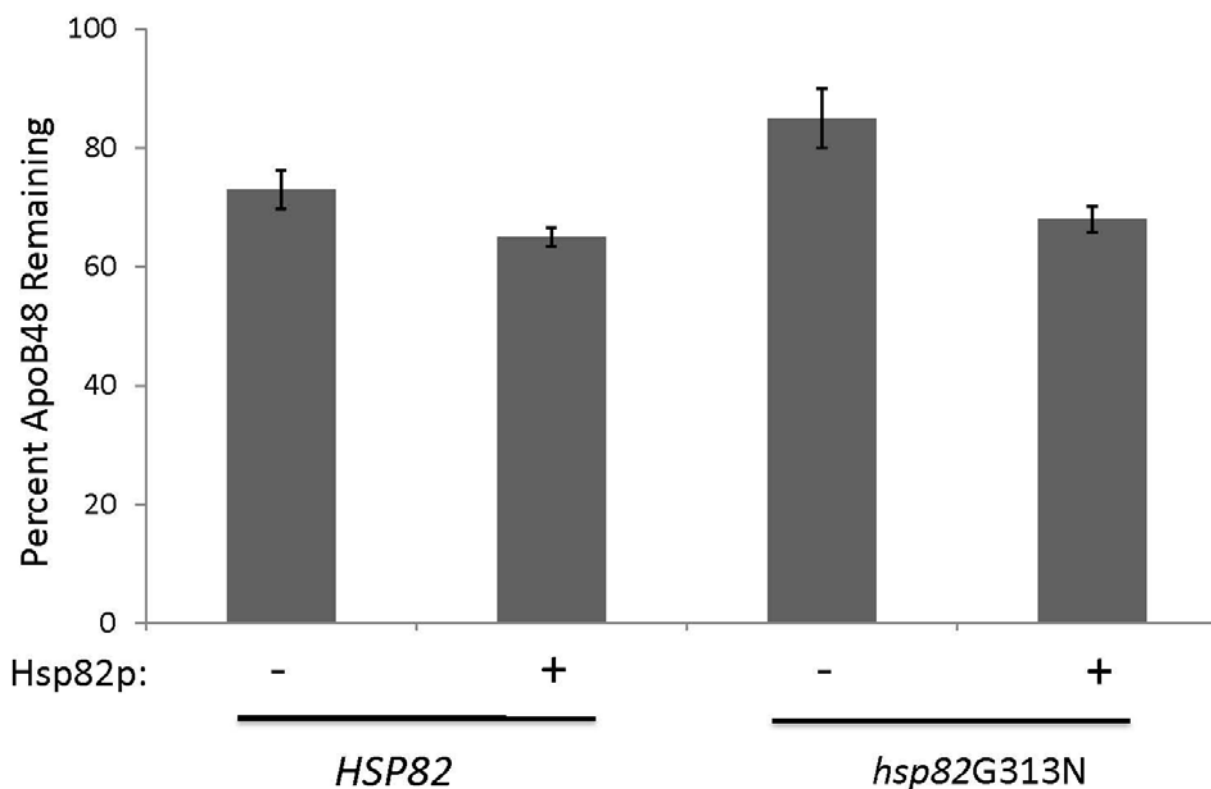
**Figure 32: LIC Peptide Does Not Impact ApoB Degradation In Vitro**

ApoB48 degradation was assessed in vitro at 37°C for 30 min using cytosol (5µg/µL) from *SSE1* or *sse1Δ* yeast with a final concentration of 250µM MG132, 50µM or 150µM LIC peptide (see text) added to the reaction. For controls an equivalent amount of DMSO or DMF were added to the reaction as the solvents were utilized to dissolve the MG132 and LIC peptide, respectively. The compounds were added to the cytosol and allowed to incubate with the reaction on ice for 15min before the reaction commenced. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO or DMF treated samples to those samples treated with MG132 or LIC peptide respectively. Data represent the means from 6 independent experiments  $\pm$  SE of the means.

levels of degradation were observed in both wildtype and *sse1Δ* cytosol in the absence and presence of an excess of peptide (Figure 32). This suggests that while the ATPase activity of Sse1p is important for the stabilization of ApoB in vivo, this peptide does not modulate the activity of Sse1p enough to alter the stabilization of ApoB by Hsp110. ApoB may have a higher binding affinity for Sse1p than the peptide and may out-compete the substrate for the binding pocket of the chaperone. In addition, this peptide does bind Ssa1p and inhibits its ATPase activity, therefore the effects of the peptide on both Sse1p (pro-stabilization) and Ssa1p (pro-degradation) may cancel each other out and as a result no effect on ApoB degradation is observed.

### **3.2.3 Hsp90, But Not The Co-Chaperones Sti1p And Sba1p Contribute To ApoB ERAD**

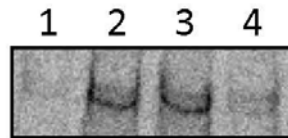
Hsp90 assists in the targeting of ApoB for ERAD and overexpression of Hsp90 results in increased ApoB degradation (Gusarova et al., 2001). I recapitulated the experiment in which the degradation of ApoB is reduced in cytosol from yeast cells expressing the temperature sensitive *HSP82* allele at the non-permissive temperature (Figure 33). In addition, I purified Hsp82p and added the chaperone into the reaction to determine if the supplementation would enhance proteasomal degradation of ApoB. The Hsp82p utilized in these assays was confirmed to be active by NBD1 aggregation assay (data not shown) (Strickland et al., 1997; Youker et al., 2004). The addition of Hsp82p to the mutant cytosol rescued the proteasome-dependent degradation of ApoB. Therefore, like Hsp110, modulating Hsp90 levels can impact ApoB degradation in vitro with yeast lysates. In addition, I recapitulated the co-immunoprecipitation



**Figure 33: Purified Hsp82p Enhances The Proteasomal Degradation Of ApoB In Vitro**

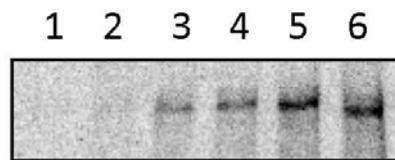
Cytosol was obtained from strains expressing a wildtype copy of Hsp82p or the G313N Hsp82p mutant that were shifted to the nonpermissive temperature of 37°C before harvesting. The cytosols were added to the degradation reaction at a final concentration of 5µg/µl and were supplemented (+) or not (-) with Hsp82p to a final concentration of 4% of the total protein. Reactions were incubated at 37°C for 30 min. Data represent the means of 6 experiments  $\pm$  SE of the mean.  $p < 0.02$

**A**



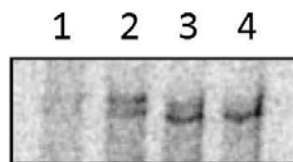
Lane #	Immunoprecipitation
1	No Antibody
2	Anti-Hsp90 + MG132
3	Anti-Hsp90 + DMSO
4	Anti-Hsp70 + MG132

**B**



Lane #	Immunoprecipitation
1	No Antibody
2	Anti-Hsp90 (0.3mg) No Cytosol
3	Anti-Hsp90 (0.1mg)
4	Anti-Hsp90 (0.3mg)
5	Anti-Hsp90 (0.6mg)
6	Anti-Hsp110 (0.6mg)

**C**



Lane #	Immunoprecipitation
1	No Antibody
2	Anti-Hsp110 (0.1mg)
3	Anti-Hsp110 (0.3mg)
4	Anti-Hsp110 (0.6mg)



**Figure 34: ApoB Co-Immunoprecipitates With Hsp90, Hsp70, And Hsp110 In Yeast Cytosol.**

- A) ApoB48 associated with microsomes was incubated in yeast cytosol at a final concentration of 10 $\mu$ g/ $\mu$ l on ice for 15min. The Hsp70 and Hsp90 were immunoprecipitated from the reaction with 3mg of the indicated anti-sera and protein A-sepharose. Radiolabeled ApoB in the precipitate was visualized by phosphorimager analyses. The addition of MG132 did not significantly increase the amount of ApoB found in the immunoprecipitate with Hsp90.
- B) ApoB48 co-immunoprecipitations were set up as described in part A. A titration with the Hsp90 antibody revealed that increasing the amount of Hsp90 antiserum can result in an increase in ApoB in the precipitate. In addition, ApoB is found in the precipitate in an immunoprecipitation reaction with Hsp110 antisera.
- C) ApoB48 co-immunoprecipitations were set up as described in part A. A titration with the Hsp110 antibody revealed that increasing the amount of Hsp110 antisera does not result in a significant increase in ApoB in the precipitate, suggesting that 0.1mg of Hsp110 antisera is sufficient to saturate the immunoprecipitation reaction.

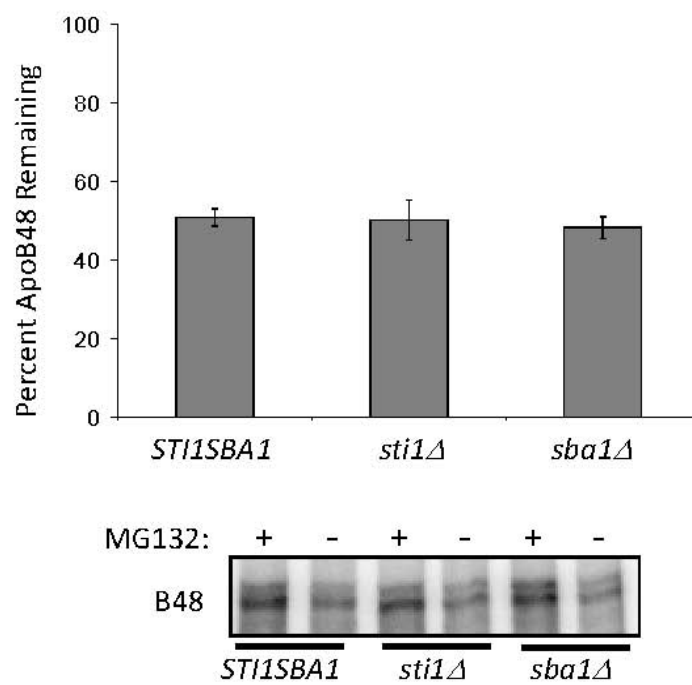
experiments with the in vitro system and detected an interaction between ApoB and Hsp82p, Ssa1p, and Sse1p (Figure 34).

As stated previously, Sse1p is a component of the Hsp90 complex and is involved in the folding of substrates associated with the Hsp90 complex (Goeckeler et al., 2002; Liu et al., 1999). In the in vivo system, I had observed distinct components of the Hsp90 complex immunoprecipitating with ApoB, Sse1p, Ssa1p, Ydj1p, Hsp82p, but not Stilp and Sba1p (the HOP and p23 homologs, respectively). Because I could detect interactions between ApoB and components of the Hsp90 complex (Ssa1p, Sse1p, Hsp82p) in vitro I wanted to determine if the Hsp90 co-chaperones, Stilp and Sba1p, contribute to ApoB degradation. I examined ApoB disappearance in cytosols from *stilΔ* and *sba1Δ* cells and as shown in Figure 35, ApoB ERAD is similar in cytosols from the wildtype and deletion strains. In addition, ApoB degradation was tested in cytosol from *sse1ΔstilΔ* cells and the extent of degradation was similar to levels observed in the cytosol from the *sse1Δ* cells.

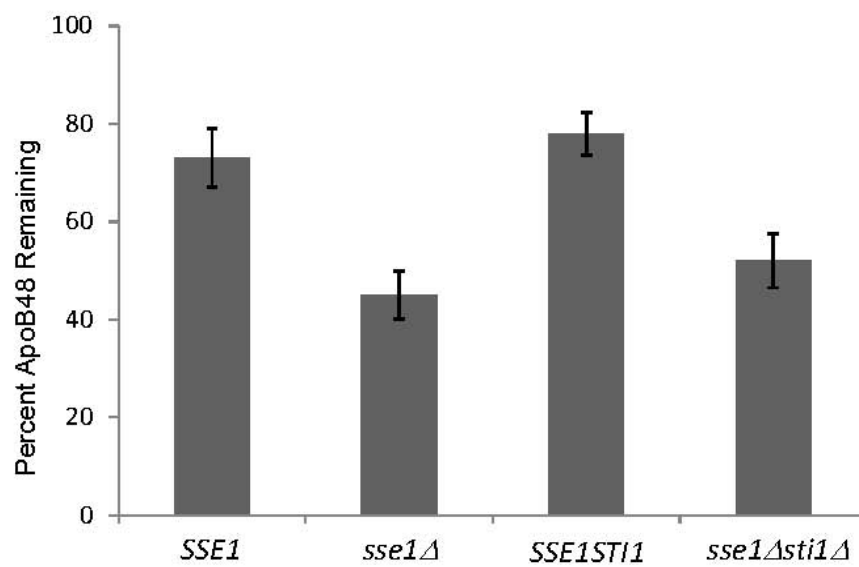
### **3.2.4 ApoB Degradation Is Not Mediated By The Disaggregase Complex**

Ssa1p is reported to function in complex with Ydj1p, Hsp26, Hsp42, and Hsp104 and participates in breaking-apart protein aggregates in order to assist with the proteasomal degradation of aggregated protein substrates (Cashikar et al., 2002; Glover and Lindquist, 1998). As both Ssa1p and Ydj1p have been shown to be pro-degradation factors for ApoB (Chapter 2.0) and ApoB is a hydrophobic, aggregation-prone protein, I wanted to examine the remaining components of this complex for their role in ApoB degradation. However, none of the mutant

**A**



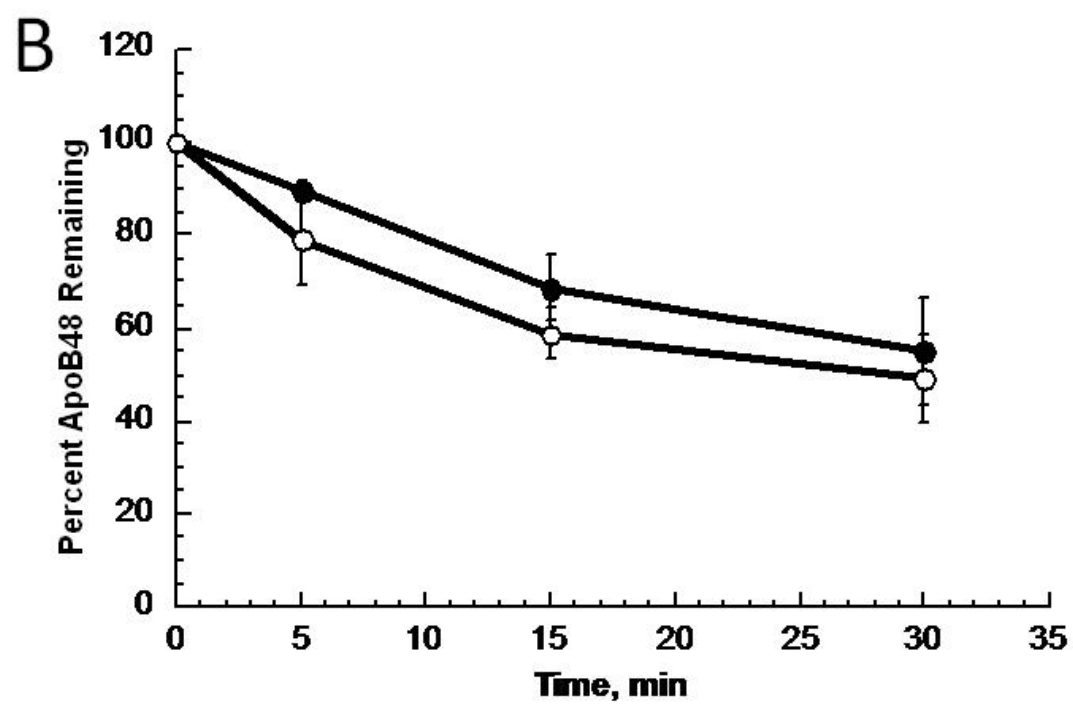
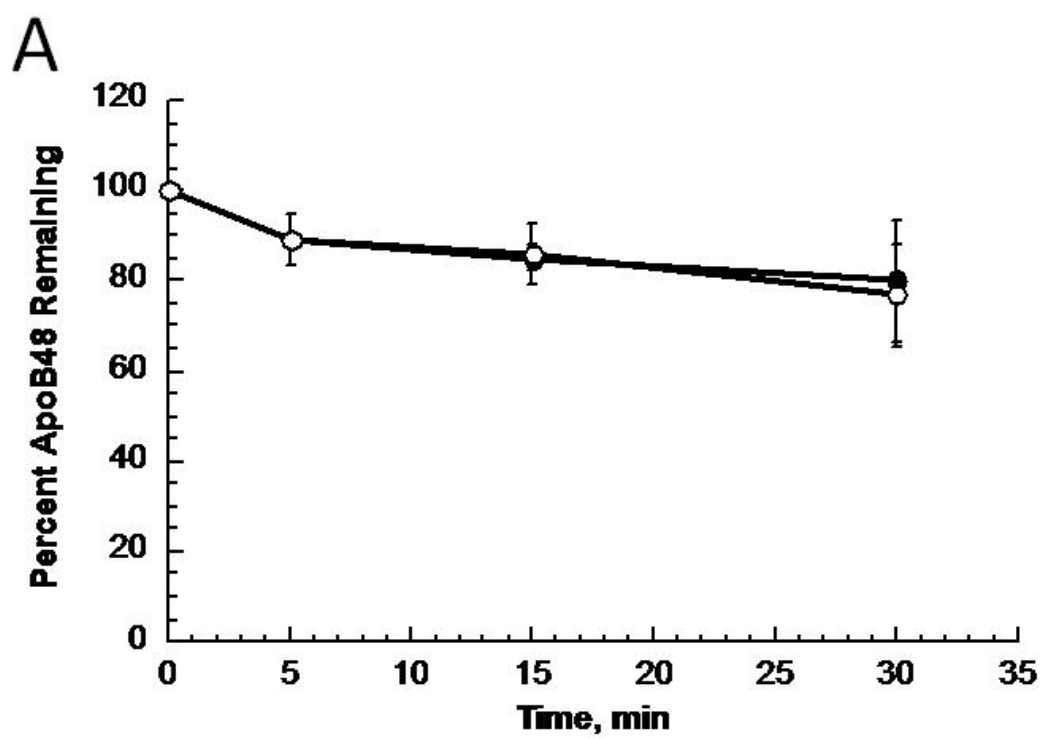
**B**



**Figure 35: ApoB Degradation Does Not Require Stilp Or Sba1p In Yeast Cytosol.**

A) ApoB48 degradation was assessed in vitro at 37°C for 30 min using cytosol (5µg/µL) from *STIISBA1*, *stilΔ* or *sba1Δ* yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 6 independent experiments  $\pm$  SE of the means. The lower panel depicts a representative phosphorimage of <sup>35</sup>S labeled ApoB48 during the degradation assay in cytosol from *STIISBA1*, *stilΔ* or *sba1Δ* yeast. Reactions were treated with the proteasome inhibitor MG132 (250µM) or DMSO (-), as indicated.

B) ApoB48 degradation was assessed in vitro at 37°C for 30min using cytosol (10µg/µL) from *STIISSE1* or *stilΔsse1Δ* yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 9 independent experiments  $\pm$  SE of the means.  $p < 0.005$



**Figure 36: ApoB Degradation Does Not Require Hsp26, Hsp42 Or Hsp104 In Vitro.**

A) ApoB48 degradation was assessed in vitro at 37°C using cytosol (5µg/µL) from *HSP26HSP42* (●) or *hsp26Δhsp42Δ* (○) yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 6 independent experiments  $\pm$  SE of the means.

B) ApoB48 degradation was assessed in vitro at 37°C using cytosol (5µg/µL) from *HSP104* (●) or *hsp104Δ* (○) yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 6 independent experiments  $\pm$  SE of the means. 30min  $p < 0.4$

cytosols tested exhibited a degradation defect for ApoB compared to the wildtype control (Figure 36).

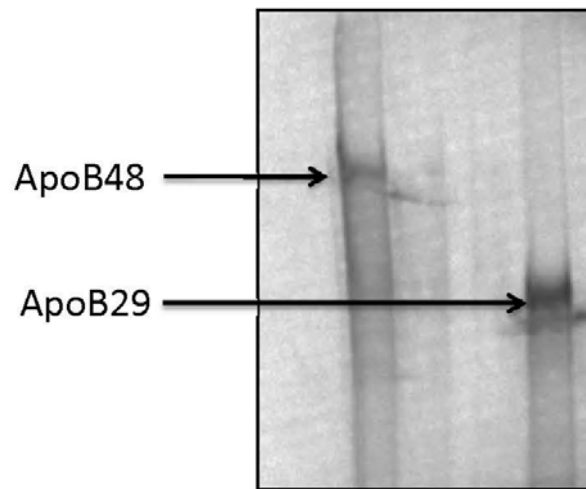
### **3.2.5 The Degradation of ApoB29 Is Enhanced In Cytosol Lacking Sse1p In Vitro**

The in vitro data were obtained using an expression construct that produces the ApoB48 isoform, a form that is expressed in the small intestine. However, my in vivo data examined the degradation and chaperone-interactions of a shorter ApoB29 isoform. In order to confirm that ApoB29 and ApoB48 function in a similar manner during proteasomal degradation I generated an ApoB29 expression construct that can be used in the in vitro system (Figure 37A). When I examined ApoB29 and ApoB48 degradation in both Sse1p wildtype and deletion cytosols I observed similar degradation for both isoforms (Figure 37B). Overall, these data suggest that these two isoforms function similarly in the in vitro degradation system.

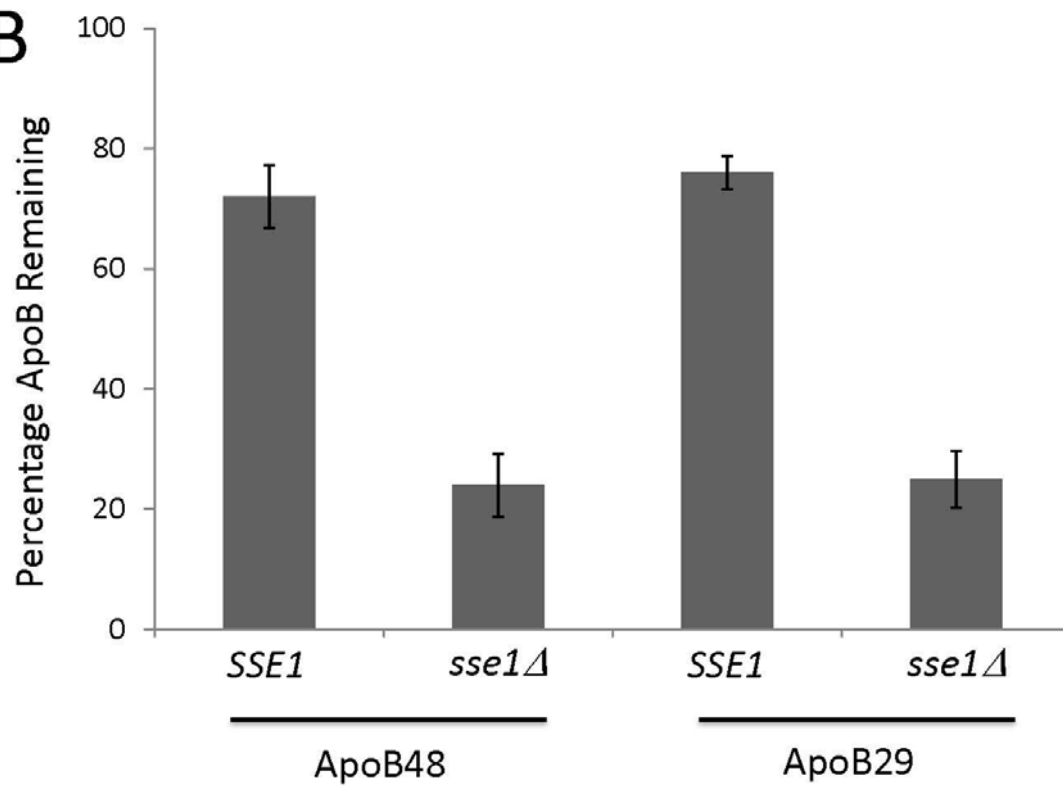
### **3.2.6 Hsp110 Overexpression Enhances ApoB Secretion In Rat Hepatoma Cells**

ApoB is normally synthesized in hepatic cells and is secreted in mature VLDL particles, which are then converted to LDL particles (Chapter 1.0). Although mammalian Hsp110 failed to improve the slow growth phenotype of *sse1Δ* yeast (K. Morano, University of Texas- Houston Medical School, personal communication) I was curious if Hsp110 might also be involved in ApoB biogenesis in hepatic cells. To test this hypothesis, I first showed that Hsp110 was present in McArdle-RH7777 cells (see below), which are a rat hepatoma cell line and an established model of ApoB metabolism, and in liver extracts (Figure 38 and Figure 39A). In addition, the cells secrete ApoB100, the form that is produced by human liver cells (Tanabe et al., 1989).

**A**



**B**

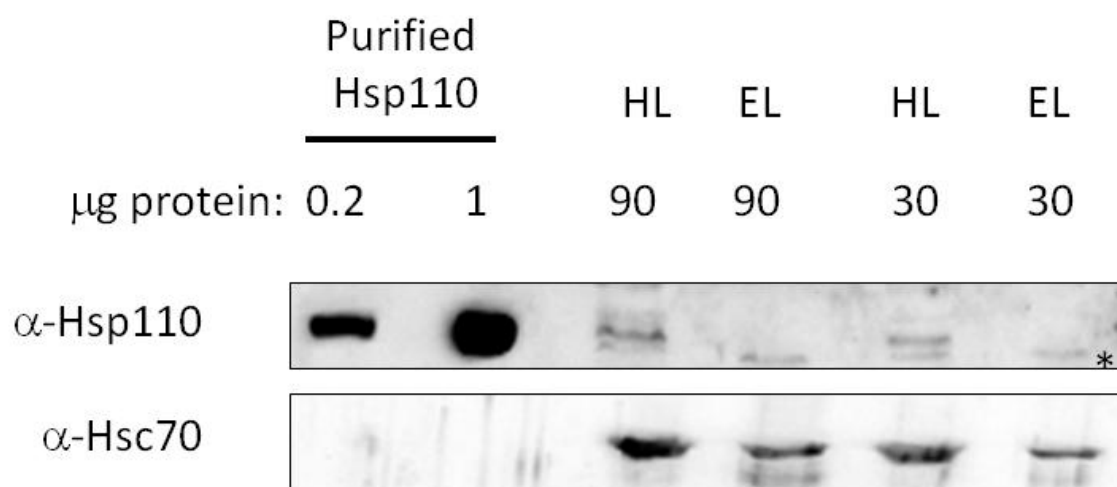




**Figure 37: ApoB48 And ApoB29 Both Exhibit Enhanced Degradation In Cytosol Lacking Sse1p**

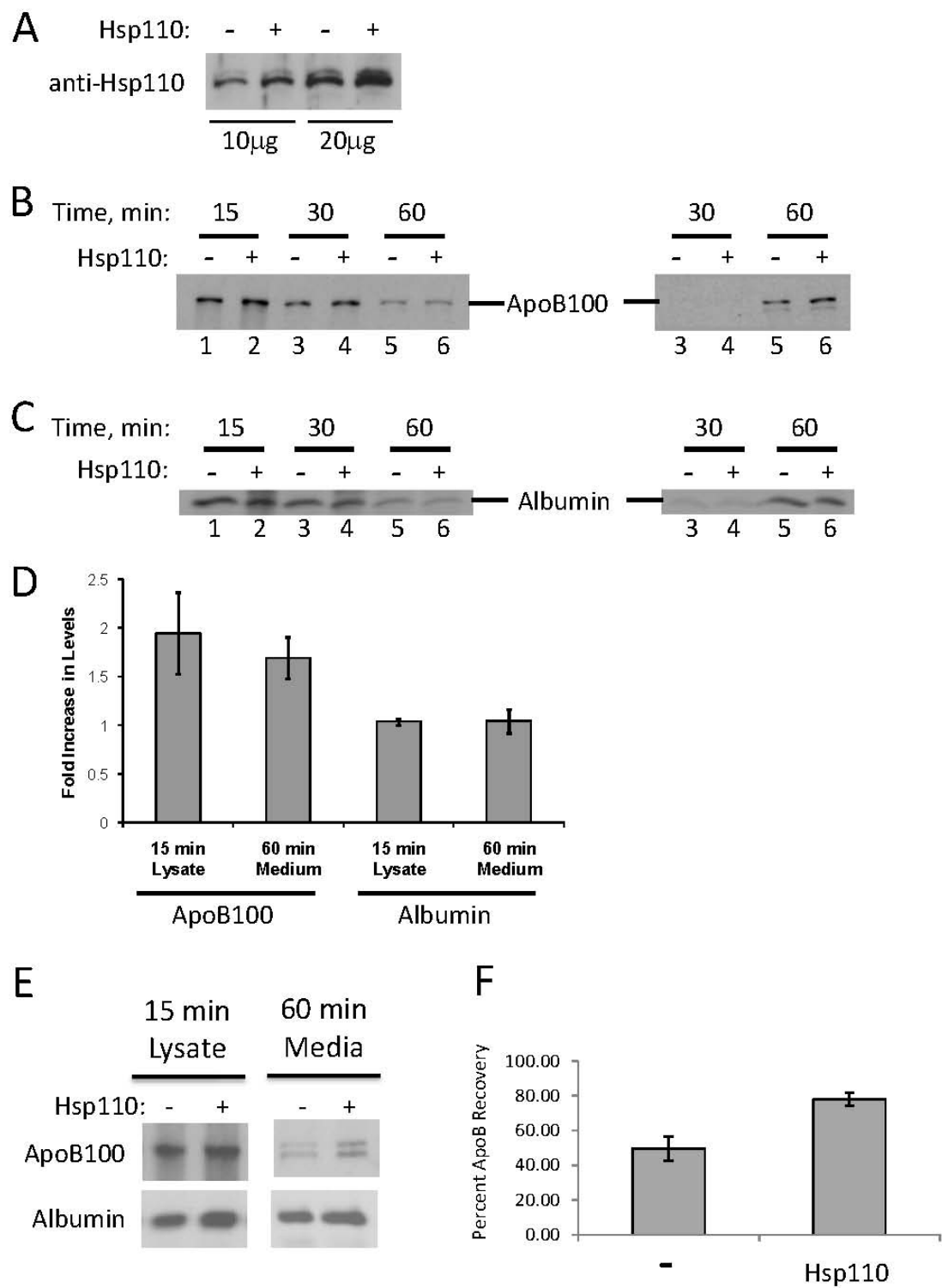
A) Approximately 2 $\mu$ l of the transcription and translation reactions of SP6-ApoB48 and SP6-ApoB29 were resolved by SDS PAGE and visualized by phosphorimager analysis.

B) ApoB48 and ApoB29 degradation were assessed in vitro at 37°C for 30min using cytosol (5 $\mu$ g/ $\mu$ L) from *SSE1* (●) or *sse1 $\Delta$*  (*JG014a*) (○) yeast. The percentage of ApoB remaining was calculated by normalizing the amount of ApoB in the DMSO treated samples to those samples treated with MG132, thus reflecting only the degree of proteasome-mediated degradation. Data represent the means from 3 independent experiments  $\pm$  SE of the means.  $p < 0.02$



**Figure 38: Hsp110 Is Expressed In Rat Hepatic Cells**

Extracts from rat hepatocytes (HL) and enterocytes (EL) were examined by western blot analysis for Hsp110 expression. Purified Hsp110 was utilized as a loading control (a kind gift from J. Subjeck, Roswell Park Cancer Institute). While Hsp110 was detected in the hepatocyte extracts, no full length Hsp110 was detected in the enterocyte lysate. However, low molecular weight bands were observed that may be degradation products of Hsp110 in the enterocyte lysate (\*). A western blot for Hsc70 was also conducted as a loading control for the extracts.



**Figure 39: ApoB Secretion Is Enhanced In Rat Hepatic Cells Overexpressing Hsp110**

A) Equal amounts of cell lysates from McArdle-RH7777 cells transfected with pcDNA3.1 or a pcDNA3.1-Hsp110 expression plasmid were analyzed by western blotting with an anti-Hsp110 antibody.

B, C) McArdle-RH7777 cells were transfected either with pcDNA3.1 (lanes 1, 3 and 5) or the pcDNA3.1-Hsp110 expression plasmid (lanes 2, 4 and 6), and 48 h after transfection cells were pulse-labeled with [<sup>35</sup>S]-methionine/cysteine for 10 min and chased in isotope-free medium for 15 (lanes 1 and 2), 30 (lanes 3 and 4) or 60 min (lanes 5 and 6). Cell lysates (left) and conditioned media samples (right) containing an equal number of trichloroacetic acid-insoluble counts per mg of total protein were immunoprecipitated with antibodies against ApoB (B) or albumin (C) and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Note that the form of ApoB displayed is ApoB100, the species secreted by a normal human liver. Data were reproducible in five complete experiments.

D) Fold increase in ApoB100 and albumin levels in McArdle-RH7777 cells transfected with pcDNA3.1-Hsp110 compared to control cells transfected with pcDNA3.1. Data represent the means from 5 independent experiments  $\pm$  SE of the means. 15 min,  $p < 0.04$ ; 60 min,  $p < 0.02$

E) McArdle-RH7777 cells were transfected either with pcDNA3.1 (lanes 1, 3 and 5) or the pcDNA3.1-Hsp110 expression plasmid (lanes 2, 4 and 6), and 48 h after transfection cells were treated with 625 $\mu$ M oleic acid, pulse-labeled with [<sup>35</sup>S]-methionine/cysteine for 10 min and chased in isotope-free medium for 15 or 60 min. Cell lysates (left) and conditioned media samples (right) containing an equal number of trichloroacetic acid-insoluble counts per mg of total protein were immunoprecipitated with antibodies against ApoB or albumin and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Note that the form of ApoB displayed is ApoB100, the species secreted by a normal human liver. Data were reproducible in three complete experiments.

F) Percent ApoB100 recovery in McArdle-RH7777 cells transfected with pcDNA3.1-Hsp110 compared to control cells transfected with pcDNA3.1. ApoB was normalized to Albumin levels in the lysate and media. The percent recovery was obtained by dividing the signal from the normalized ApoB in the media by the normalized ApoB in the lysate. Data represent the means from three independent experiments  $\pm$  SE of the means.  $p < 0.02$

Next, the cells were transfected with an Hsp110 over-expression plasmid or with a control vector and a pulse-chase analysis was performed. I initially observed that Hsp110 expression increased only 1.8-fold in cells transformed with the Hsp110 expression vector (Figure 39A) (Data for Figure 39A-D was obtained by V. Gusarova, S.L.H., J.L.B., and E.A. Fisher), suggesting that the effect on ApoB—if any—would be subtle. Nevertheless, I observed 1.9-fold more ApoB intracellularly at the 15 min time point, an effect that translated into an 1.7-fold increase in ApoB secretion after 60 min (Figure 39B,D). The lack of ApoB in the conditioned medium samples at the 30 min time point (Figure 39B) was expected based on previous data that it takes approximately 40 min for newly synthesized ApoB to be secreted from hepatic cells (Borchardt and Davis, 1987). As a control for this experiment, I found that the levels and secretion of albumin, another protein synthesized in hepatic cells, was unaffected regardless of whether Hsp110 was over-expressed (Figure 39C,D). In addition, when I examined ApoB recovery in Hsp110 over-expressing cells and control cells in the presence of oleic acid, a compound that stimulates ApoB secretion, I observed an increase in the percent of ApoB recovered in the lysate and the medium fractions from 49.6% (control) to 78% (upon Hsp110 overexpression) (Figure 38E,F). Furthermore, I found that the over-expression of Hsp110 in these cells in the presence of an MTP inhibitor (BMS-200150) (Jamil, 1996) increased the percent of ApoB recovered in the lysate and medium fractions from 14% to 28% (Data not shown) (Data by D. Habel, S.L.H., J.L.B., and E.A. Fisher).

### **3.2.7 Brief Summary Of In Vitro Results**

Overall, the results obtained with the in vitro system confirmed that ApoB29 and ApoB48 have similar chaperone requirements for degradation. Hsp110 is a pro-stabilization factor for ApoB both in vivo and in vitro. The role for Hsp110 in ApoB maturation and secretion was confirmed in a rat hepatoma cells line. The stabilization of ApoB by the overexpression of Hsp110 in the experiments using a mammalian cell culture system was independent of MTP activity and lipid content. Attempts to modulate the effect of Hsp110 on ApoB degradation with a peptide known to stimulate its ATPase activity were unsuccessful, however hopefully future experiments will isolate a compound that modulates Hsp110 activity and has potential therapeutic benefits for patients with high circulating levels of cholesterol.

**Table 4: Yeast Strains Utilized In This Study**

<b>Strain</b>	<b>Genotype</b>	<b>Reference</b>
<i>W3031b</i>	<i>MAT<math>\alpha</math>, ade2, his3, leu2, trp1, ura3, can1-100</i>	Shirayama, M, <i>et al.</i> 1993
<i>SSE1 (JG014a)</i>	<i>MAT<math>\alpha</math>, his3, leu2, trp1, ura3</i>	Goeckeler, JG, <i>et al.</i> 2002
<i>sse1<math>\Delta</math> (JG014a)</i>	<i>MAT<math>\alpha</math>, his3, leu2, trp1, sse1::URA3</i>	Goeckeler, JG, <i>et al.</i> 2002
<i>SSE1 (E0020)</i>	<i>MAT<math>\alpha</math>, his3, leu2, ura3</i>	Shirayama, M, <i>et al.</i> 1993
<i>sse1<math>\Delta</math> (E0020)</i>	<i>MAT<math>\alpha</math>, his3, leu2, ura3, sse1::HIS3</i>	Shirayama, M, <i>et al.</i> 1993
<i>sse1<math>\Delta</math> (w303)</i>	<i>MAT<math>\alpha</math>, ade2-1, can1-100, his2-11, 15, leu2-3, 112, trp1-1, ura2-1, SSE1::Kan</i>	Shaner, L, <i>et al.</i> 2004
<i>SSB1SSB2</i>	<i>MAT<math>\alpha</math>, his3, leu2, lys2, trp1, ura3</i>	Nelson, RJ, <i>et al.</i> 1992
<i>ssb1<math>\Delta</math> ssb2<math>\Delta</math></i>	<i>MAT<math>\alpha</math> his3, leu2, lys2, trp1, ura3, ssb1-1, ssb2-1</i>	Nelson, RJ, <i>et al.</i> 1992
<i>CIM3</i>	<i>MAT<math>\alpha</math>, ura3-52, his3<math>\Delta</math>200, leu2-<math>\Delta</math>1</i>	Ghislain, M, <i>et al.</i> 1993
<i>cim3-1</i>	<i>MAT<math>\alpha</math>, cim3-1, ura3-52, his3<math>\Delta</math>200, leu2-<math>\Delta</math>1</i>	Ghislain, M, <i>et al.</i> 1993
<i>PEP4</i>	<i>MAT<math>\alpha</math>, can1-100, leu2-3, 112, his3-11, trp1-1, ura3-1, ade2-1</i>	Jones, EW, <i>et al.</i> 1982
<i>pep4<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, PEP4::TRP1, leu2-3, 112, his3-11, trp1-1, ura3-1, ade2-1</i>	Jones, EW, <i>et al.</i> 1982
<i>KAR2</i>	<i>MAT<math>\alpha</math>, ura3-52, leu2-3, 112, ade2-101</i>	Brodsky, JL, <i>et al.</i> 1999
<i>kar2-1</i>	<i>MAT<math>\alpha</math>, ura3-52, leu2-3, 112, ade2-101, kar2-1</i>	Brodsky, JL, <i>et al.</i> 1999
<i>UBC6UBC7</i>	<i>MAT<math>\alpha</math>, lys2-801, his3<math>\Delta</math>200, ura3-52, trp1-1, leu2-3, 112</i>	Chen, P, <i>et al.</i> 1993
<i>ubc6<math>\Delta</math>ubc7<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, lys2-801, his3<math>\Delta</math>200, ura3-52, trp1-1, leu2-3, 112, ubc6::HIS3, ubc7::LEU2,</i>	Chen, P, <i>et al.</i> 1993
<i>UFD1</i>	<i>MAT<math>\alpha</math>, his4-519, ura3-52, ade1-100, leu2-3, 112</i>	Ye, Y, <i>et al.</i> 2001
<i>ufd1-1</i>	<i>MAT<math>\alpha</math>, his4-519, ura3-52, ade1-100, leu2-3, 112, ufd1-1</i>	Ye, Y, <i>et al.</i> 2001
<i>DOA10HRD1</i>	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3, 112, ura3-52, lys2-801, trp1-1</i>	Swanson, R, <i>et al.</i> 2001
<i>doa10<math>\Delta</math>hrd1<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3, 112, ura3-52, lys2-801, trp1-1, doa10::HIS3, hrd1::LEU2</i>	Swanson, R, <i>et al.</i> 2001

Strain	Genotype	Reference
UBC7	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3,112, ura3-52, lys2-801, trp1-1</i>	Chen, P, <i>et al.</i> 1993
<i>ubc7<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc7::LEU2</i>	Chen, P, <i>et al.</i> 1993
<i>ubc6<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc6::HIS3</i>	Chen, P, <i>et al.</i> 1993
<i>sti1<math>\Delta</math></i> (w303)	<i>MAT<math>\alpha</math>, ade2, his3, leu2, trp1, ura3, can1-100, sti1::HIS3</i>	Ghislain, M, <i>et al.</i> 1993
<i>sba1<math>\Delta</math></i> (w303)	<i>MAT<math>\alpha</math>, ade2, his3, leu2, trp1, ura3, can1-100, sba1::URA3</i>	Ghislain, M, <i>et al.</i> 1993
BY4742	<i>MAT<math>\alpha</math>, his3, leu2, lys2, ura3</i>	Scott, C, <i>et al.</i> 2007
<i>add66<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3, leu2, lys2, ura3, add66::KANMX</i>	Scott, C, <i>et al.</i> 2007
SCJ1 JEM1	<i>MAT<math>\alpha</math>, his3, leu2, lys2, trp1, ura3, suc2-<math>\Delta</math>9</i>	Nishikawa, S, <i>et al.</i> 1997
<i>scj1<math>\Delta</math>jem1<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3, leu2, lys2, trp1, ura3, suc2-<math>\Delta</math>9, jem1::LEU2, scj1::TRP1</i>	Nishikawa, S, <i>et al.</i> 1997
SNL1FES1	<i>MAT<math>\alpha</math>, ade2, his3, leu2, lys2, trp1, ura3</i>	Provided by JasonYoung
<i>snl1<math>\Delta</math>fes1<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, ade2, his3, leu2, lys2, trp1, ura3, fes1::KANMX, snl1::HIS3</i>	Provided by Jason Young
STI1 SSE1	<i>MAT<math>\alpha</math>, GAL2, his3-11,15 leu2-3,112, lys1, lys2, trp1<math>\Delta</math>, ura3-52</i>	Liu, XD, <i>et al.</i> 1999
<i>sti1<math>\Delta</math>sse1<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, GAL2, his3-11,15 leu2-3,112, lys1, lys2, trp1<math>\Delta</math>, ura3-52, sti1::HIS3, sse1::KAN</i>	Liu, XD, <i>et al.</i> 1999
HSP104	<i>MAT<math>\alpha</math> ade2-101, his3-<math>\Delta</math>200, leu2-<math>\Delta</math>1, lys2-801, trp1-<math>\Delta</math>63, ura3-52</i>	Lum, R, <i>et al.</i> 2004
<i>hsp104<math>\Delta</math></i>	<i>MAT<math>\alpha</math> ade2-101, his3-<math>\Delta</math>200, leu2-<math>\Delta</math>1, lys2-801, trp1-<math>\Delta</math>63, ura3-52, hsp104::LEU2</i>	Lum, R, <i>et al.</i> 2004
HSP26HSP42	<i>MAT<math>\alpha</math> ade2-101, his3-<math>\Delta</math>200, leu2-3,112, suc2-<math>\Delta</math>9, trp1-<math>\Delta</math>901, ura3-52</i>	Haslbeck, M, <i>et al.</i> 2004
<i>hsp26<math>\Delta</math>hsp421<math>\Delta</math></i>	<i>MAT<math>\alpha</math> ade2-101, his3-<math>\Delta</math>200, leu2-3,112, suc2-<math>\Delta</math>9, trp1-<math>\Delta</math>901, ura3-52, hsp26::HIS3, hsp42::LEU2</i>	Haslbeck, M, <i>et al.</i> 2004
<i>hsp82-G313N</i>	<i>MAT<math>\alpha</math> ade2-101, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPDH-HSP82-G313N (TRP1)</i>	Bohen, SP, <i>et al.</i> 1993



**Table 5: Antisera Utilized In This Study**

<b>Antibody</b>		<b>Source</b>
anti-ApoB (1D1)	Mouse monoclonal	R. Milne
anti-Albumin	Sheep polyclonal	Bethyl Laboratories
anti-Kar2p	Rabbit polyclonal	R. Schekman
anti-HA	Mouse monoclonal	Roche
anti-Hsp82p	Rabbit polyclonal	A. Caplan
anti-L3	Mouse monoclonal	J. Warner
anti-mammalian Hsp110	Rabbit polyclonal	Stressgen
anti-Met19p	Rabbit polyclonal	Sigma
anti-PKC1p	Rabbit polyclonal	Santa Cruz Biotechnology
anti-Sba1p	Rabbit polyclonal	A. Caplan
anti-Sec61p	Rabbit polyclonal	R. Schekman
anti-Sec63p	Rabbit polyclonal	D. Feldheim
anti-Ssa1p	Rabbit polyclonal	J. Brodsky
anti-Ssb1p	Rabbit polyclonal	E. Craig
anti-Sse1p	Rabbit polyclonal	J. Brodsky
anti-Sti1p	Mouse monoclonal	D. Toft
anti-Tim23p	Rabbit polyclonal	T. Endo
anti-Ydj1p	Rabbit polyclonal	A. Caplan
anti-Myc	Mouse monoclonal	S. Michaelis

**Table 6: Plasmids Utilized In This Study**

Plasmid	Reference
pSLW1	This study
pSLW1-B29HA	This study
p414TEF	Shaner et al., 2004
p414TEFSSE1	Shaner et al., 2004
p414TEFSSE1K69Q	Shaner et al., 2004
p414TEFSSE1G205D	Shaner et al., 2004
p414TEFSSE1G233D	Shaner et al., 2004
p414TEFSSE1-CTD(394-693)	Shaner et al., 2004
pRS426-CFTR-HA	Zhang, et al., 2001
pSM36-ppafDG-HA	Kim, et al., 2005
pRS315-CPY*-HA	Ng, et al., 2000
pSM1082-Ste6p*-HA	Loayza, et al., 1998
pSP6-B48	Gusarova, et al., 2001
pSP6-B29	This study
pSM989 (Ub-Myc)	Ellison and Hochstrasser, 1991
pSM990 (Ub-HA)	Hochstrasser, et al. 1991

## 4.0 DISCUSSION

Data from yeast expression systems with various mammalian substrates have shown that chaperones play an important role in the degradation of the proteins (Ahner et al., 2007; McClellan et al., 2005; Palmer et al., 2003; Ravid et al., 2006; Youker et al., 2004; Zhang et al., 2001). In addition, the results obtained with yeast can often be recapitulated in mammalian systems (Ahner et al., 2007; Gusarova et al., 2001; Jiang et al., 1998; Loo et al., 1998; Meacham et al., 1999). When the chaperone and UPP requirements of various ERAD substrates are compared, it becomes evident that each substrate requires a unique subset of molecular chaperones and related factors to regulate the maturation or degradation of the polypeptide (for a recent review see (Brodsky, 2007)). Previous work has established ApoB as a unique ERAD substrate that undergoes co-translational degradation (Benoist and Grand-Perret, 1997; Du et al., 1999; Fisher et al., 1997; Gusarova et al., 2001; Liang et al., 2003; Liao et al., 1998; Linnik and Herscovitz, 1998; Sakata and Dixon, 1999; Tatu and Helenius, 1999; Yeung et al., 1996; Zhang and Herscovitz, 2003; Zhou et al., 1998). Prior to my work, Hsp70 and Hsp90 were the only cytosolic chaperones known to be important for targeting ApoB for ERAD (Fisher et al., 1997; Gusarova et al., 2001; Zhou et al., 1998; Zhou et al., 1995). One major goal of this dissertation was to develop a yeast expression system to further characterize the chaperone requirements for ApoB ERAD. To this end, I successfully expressed a human ApoB isoform in yeast and determined that it associates with the ER and requires components of the UPP for degradation.

Utilizing this system I then examined the contributions of various molecular chaperones on ApoB ERAD and identified Hsp110 as the first pro-stabilization chaperone for ApoB. A second major goal of this dissertation was to utilize a previously described in vitro system to confirm and extend the results obtained with the in vivo system. Using this system, I was able to reduce or enhance the degradation of ApoB by supplementing cytosol with purified individual chaperones. The final goal of this dissertation was to confirm that Hsp110 is a pro-stabilization factor and promotes the secretion of ApoB in a mammalian cell culture system. I found that the stabilization and increased secretion of ApoB in cells overexpressing Hsp110 was independent of MTP activity and oleic acid stimulation, suggesting that this molecular chaperone participates in ApoB maturation in mammalian cells irrespective of the metabolic status of the cell.

#### **4.1 APOLIPOPROTEIN B IS AN ERAD SUBSTRATE IN YEAST**

Three main ERAD pathways (ERAD-L, ERAD-C, and ERAD-M) have been characterized based on the chaperone and UPP component requirements for the degradation of several model substrates (see section 1.2.1). While ERAD-L and ERAD-C have been well characterized, relatively little is known about ERAD-M. ApoB is an unusual ERAD substrate as it is targeted for degradation while being translated and is in a bi-topic orientation in the translocon (Fisher and Ginsberg, 2002). ApoB is an amphipathic polypeptide with regions of hydrophobicity critical for its lipid binding capacity and hydrophilic regions that are important for maintaining the solubility of the lipoprotein particle in serum (Segrest et al., 1994). Due to its unusual composition and the unique manner in which it is subjected to ERAD, it is not known if ApoB is more similar to an ERAD-L or ERAD-C substrate. I discovered that the ERAD of ApoB

requires both cytosolic and luminal factors (Table 7). Therefore, ApoB appears to require components of both the ERAD-L and ERAD-C pathways, further positioning ApoB as an ERAD substrate with unique attributes.

ApoB only requires the E2 ubiquitin conjugating enzyme, Ubc7p, for its degradation in yeast while another E2 associated with ERAD, Ubc6p, is dispensible for ApoB degradation (Figure 20). This is a feature associated with ERAD-L substrates (Vashist and Ng, 2004). This is particularly interesting because the known E3 ubiquitin ligase for ApoB in mammalian cells, gp78, is a functional partner for the mammalian homolog of Ubc7p (Fang et al., 2001; Liang et al., 2003). In the future, it would be interesting to determine if the polyubiquitination of ApoB is dependent upon Ubc7p in yeast and the E2 homolog in mammalian cells. In addition, further examination of the E3 requirements of ApoB degradation in yeast would be intriguing. My data indicated that ApoB is stabilized in a *hrd1Δdox10Δ* strain. However, if ApoB is an ERAD-L substrate in yeast it should only require Hrd1p for polyubiquitination and degradation (Vashist and Ng, 2004). If ApoB degradation relies predominantly on Hrd1p in yeast then the mammalian homologs (Kikkert et al., 2004; Nadav et al., 2003) should be examined to determine if they too participate in the polyubiquitination of ApoB. The action of several E3s during ERAD in mammals has been observed for other substrates such as CFTR (Meacham et al., 2001; Morito et al., 2008; Younger et al., 2004).

I also found that the ERAD of ApoB was attenuated in yeast containing a thermosensitive mutation in the gene encoding Kar2p (BiP) (Figure 24), a luminal Hsp70 chaperone that associates with ApoB (Adeli et al., 1997; Linnik and Herscovitz, 1998; Rashid et al., 2002; Zhang and Herscovitz, 2003). Interestingly, Scj1p and Jem1p, the Kar2p cognate Hsp40s

**Table 7: Mutations In The Following Proteins And Their Effects On ApoB Degradation**

<b>Protein</b>	<b>Function</b>	<b>Effect on ApoB Degradation in the Presence of Cytosol from Yeast Lacking or Expressing a Mutant Version of the Indicated Protein(s)</b>	<b>Effect on ApoB Degradation in Yeast Cells Lacking or Expressing a Mutant Version of the Indicated Protein(s)</b>
Add66p	Proteasome Assembly Factor	None	N/A
Cim3p	Proteasome Subunit in 19s Cap	N/A	Stabilized
Pep4p	Vacuolar Protease	N/A	None
Ubc6p	E2 Ubiquitin Conjugating Enzyme	N/A	None
Ubc7p	E2 Ubiquitin Conjugating Enzyme	N/A	Stabilized
Doa10pHrd1p	E3 Ubiquitin Ligases	N/A	Stabilized
Ufd1p	Component of Cdc48p Complex	Stabilized	Stabilized
Cdc48p	Component of Cdc48p Complex	Stabilized	N/A
Ssa1p	Cytosolic Hsp70	Stabilized	N/A
Ssb1pSsb2p	Cytosolic Ribosome Associated Hsp70	None	None
Ydj1pHlj1p	Cytosolic Hsp40s	N/A	Stabilized
Hsp104p	Cytosolic disaggregase	None	N/A
Hsp26pHsp42p	Cytosolic chaperones	None	N/A
Hsp82p	Cytosolic Hsp90	Stabilized	N/A
Snl1pFes1p	Nucleotide exchange factor for Ssa1p	N/A	None
Sti1p	Hsp90 Co-chaperone	N/A	None
Sba1p	Hsp90 Co-chaperone	N/A	None
Sse1p	Cytosolic Hsp110	Enhanced	Enhanced
Kar2p	ER Luminal Hsp70	N/A	Stabilized
Scj1pJem1p	ER luminal Hs40s	N/A	None

reported to function with Kar2p in ERAD-L (Nishikawa et al., 2001), had no impact on ApoB degradation. Given the large, lipophilic N-terminal domains of ApoB that reside in the ER it is not surprising that BiP may augment the retro-translocation competence of ApoB. BiP facilitates the degradation of some ERAD substrates because it prevents the aggregation of ER luminal polypeptides prior to their retro-translocation (Kabani et al., 2003). These data have been supported by some studies using mammalian cells. Specifically, ApoB turnover was enhanced in McA-RH7777 rat hepatoma cells treated with glucosamine, a compound that causes an increase in ER chaperone levels (including BiP), and when BiP was overexpressed by treatment with an adenoviral expression system (Qiu et al., 2004). However, these experiments did not examine the levels of other chaperones in response to BiP upregulation and it is not clear if the overexpression of the ER luminal Hsp70 caused ER stress that induced the UPR. These caveats should be addressed. In addition, BiP is involved in several cellular processes beyond catalyzing protein folding and ERAD (Alder et al., 2005; Denic et al., 2006; Dudek et al., 2002; Kabani et al., 2003; Nishikawa et al., 2001; Taxis et al., 2003). For example, BiP is important for both co-translational and post-translational translocation of polypeptides into the ER lumen (Bies et al., 1999; Brodsky et al., 1995; Brodsky and Schekman, 1993; Dudek et al., 2002; Matlack et al., 1999; Young et al., 2001; Zhang and Herscovitz, 2003). Therefore, altering levels of BiP may cause secondary effects that impact ApoB degradation.

BiP silencing has also been utilized to determine that the Hsp70 is required for the degradation of MHC class I heavy chain in mammalian cells (Hegde et al., 2006). In these studies, the UPR was induced by BiP silencing, however the authors showed the effect of BiP silencing on substrate turnover was distinct compared to substrate turnover in cells treated with a compound that induces the UPR. Therefore, ApoB turnover could be examined in cells in which

BiP expression has been silenced. It would subsequently be important to determine if lowering the levels of BiP results in a change in translocation efficiency for ApoB. As mentioned previously, since BiP has been suggested to function as a molecular ratchet for the translocation of polypeptides (Bies et al., 1999; Brodsky et al., 1995; Brodsky and Schekman, 1993; Dudek et al., 2002; Matlack et al., 1999; Young et al., 2001), a BiP-ApoB interaction may be critical for efficient ApoB translocation. The MTP-PDI heterocomplex may simultaneously ratchet ApoB into the ER (Benoist and Grand-Perret, 1997; Cardozo et al., 2002; Jamil, 1996; Liao et al., 2003; Mitchell et al., 1998). Ideally, the isolation and expression of BiP mutants in mammalian cells that have distinct ERAD and translocation defects could be used to examine the role of this chaperone in distinct steps during ApoB maturation and degradation.

Recently, a mammalian J domain containing protein, p58<sup>IPK</sup>, has been shown to be required for efficient co-translational degradation of ApoB and is reported to interact with BiP (Oyadomari et al., 2006; Rutkowski et al., 2007). As there is no p58<sup>IPK</sup> homolog in yeast, these data cannot be confirmed with my in vivo expression system. I have expressed p58<sup>IPK</sup> in yeast, however it is unclear if it is oriented properly in the ER membrane as p58<sup>IPK</sup> was susceptible to proteinase K digestion in microsomes from p58<sup>IPK</sup> expressing yeast (data not shown). However, if a p58<sup>IPK</sup> construct that localizes to the ER lumen in yeast is developed subsequent experiments could probe the interactions of p58<sup>IPK</sup> with BiP. In addition, the effect of p58<sup>IPK</sup> expression on ApoB translocation and ERAD in yeast could also be examined. Regardless of the success of p58<sup>IPK</sup> expression in yeast, it would be interesting to determine if p58<sup>IPK</sup> helps recruit BiP to ApoB in a mammalian system by assessing ApoB-BiP interactions in p58<sup>IPK</sup> <sup>-/-</sup> cells (Oyadomari et al., 2006; Rutkowski et al., 2007). In reciprocal experiments, the ApoB-p58<sup>IPK</sup> interactions could also be examined in cells with altered levels of BiP.



Other ERAD-L components that have been precipitated with ApoB are the ER-luminal chaperones calnexin and protein disulfide isomerase (PDI) (Linnik and Herscovitz, 1998; Tatu and Helenius, 1999). Calnexin has been reported to interact with ApoB, a protein that is highly glycosylated upon entrance into the ER (Harazono et al., 2005). Calnexin is a lectin that retains polypeptides in the ER and assists with the folding of the protein until the final glucose residue is cleaved and the properly folded protein can exit the ER (see above, Figure 12). Previous work has examined the degradation of ApoB in cells treated with compounds that disrupt the glycosylation status of ApoB. While an interaction between ApoB and calnexin could be detected in COS cells, subsequent treatment of COS cells with tunicamycin, a compound that inhibits glycosylation (Elbein, 1987), or overexpression of calnexin by transient transfection did not alter ApoB degradation (Patel and Grundy, 1996). In addition, when the authors co-expressed ApoB and MTP in the monkey kidney cell line that does not endogenously express the ER luminal factor, the ApoB-calnexin interaction was abolished. However, these data contradict studies in hepatic cell lines that endogenously express MTP. For example, when the human hepatocancer cell line, HepG2, cells were treated with tunicamycin, ApoB ubiquitination and turnover were enhanced (Liao and Chan, 2001; Macri and Adeli, 1997a; Macri and Adeli, 1997b). Yet, no difference in ApoB translocation efficiency was observed in the treated cells (Macri and Adeli, 1997b). ApoB and calnexin interactions are also observed in McRH7777 cells, a rat hepatic cell line (Tatu and Helenius, 1999). The authors also demonstrated that the interaction between ApoB and calnexin can be disrupted by treatment with castanospermine, a compound that blocks glucose trimming by both  $\alpha$  and  $\beta$  glucosidases (Elbein, 1991). These results were recapitulated in HepG2 cells where treatment with castanospermine decreased ApoB-calnexin interactions (Chen et al., 1998). Furthermore, the authors reported increased

ApoB turnover in the treated cells. Therefore, the studies in hepatic cell lines that endogenously express MTP, the disruption the glycosylation status of ApoB with compounds can alter the functional interaction of ApoB with calnexin and alter ApoB ubiquitination and turnover (Chen et al., 1998; Liao and Chan, 2001; Macri and Adeli, 1997a; Macri and Adeli, 1997b; Tatu and Helenius, 1999). While calnexin knock out mice are not viable, their work provided a cell line that expressed a truncation of calnexin that removed essential residues for the function of the protein (Denzel et al., 2002). Future studies to address the role of calnexin in ApoB maturation and turnover could utilize this cell line as it exhibited reduced folding of another glycosylated substrate in the ER (Molinari et al., 2004).

Importantly, the glycosylation-rich region of ApoB is found on the extreme C-terminus of the protein, and therefore these sites would not be exposed to the ER lumen until late in polypeptide synthesis. The two isoforms I work with in the in vitro (ApoB48) and in vivo (ApoB29) systems have fewer glycosylation sites than the full length ApoB100 protein (five and two sites, respectively, see above, Figure 2). However, as the studies in mammalian cells rely on treatment of cells with castanospermine and tunicamycin, two compounds that induce the UPR, and a complete calnexin *-/-* cell line is not available, future examination of ApoB degradation in the yeast system would be beneficial. Because microsomes that are used in the in vitro system are not amenable to genetic manipulation, the role of calnexin in ApoB ERAD in yeast would best be examined with the in vivo system. I was not able to detect glycosylation of ApoB29 in the in vivo yeast system by endoglycosidase H treatment (data not shown). However, if ApoB29 exhibits an ERAD defect in *cne1Δ* cells, mutagenesis of the glycosylation sites encoded by the polypeptide could be conducted. The degradation of the mutated ApoB proteins could be

examined in the *cne1Δ* strain and Cne1p-ApoB mutant interactions could be probed by immunoprecipitation to determine if the sites are critical for the protein-chaperone interactions.

PDI is another chaperone that is linked to the earlier steps of ApoB biogenesis. Not only is PDI found in the mammalian MTP heterocomplex that lipidates nascent ApoB as it enters the ER, but the first N-terminal 1000 amino acid domain of ApoB is highly disulfide bonded (Mitchell et al., 1998; Tietge et al., 1999; Yang et al., 1990). These disulfide bonds have been shown to be critical for ApoB secretion in mutagenesis experiments (Burch and Herscovitz, 2000). However, a direct link between the chaperone function of PDI and ApoB ERAD has not been established. As the yeast ER contains only 5 PDIs, compared to at least 17 in the mammalian ER (Buck et al., 2007; van Anken and Braakman, 2005), this model organism would be a good system to examine ApoB degradation in cells with different PDI compositions. In addition, strain with genomic deletions of all five PDI genes can be rescued with the overexpression of individual PDIs, allowing researchers to examine the contributions of individual PDIs on substrate maturation and degradation (Norgaard et al., 2001). Once the contribution of the individual PDIs on wildtype ApoB turnover have been examined, the cysteine residues in ApoB could be mutated and the degradation of the mutant ApoB proteins could be subsequently analyzed in the PDI mutant strains. This may reveal critical disulfide bonds that are instrumental in targeting ApoB for ERAD. In the future, attempts to express MTP in yeast may yield a system in which the protein interacts with PDI in the yeast ER. If this were attained, the subsequent experiments co-expressing ApoB with MTP in yeast could determine if the solubility and density of ApoB changes in the the presence of MTP. Finally, the addition of lipids to ApoB in the yeast ER may facilitate secretion of ApoB from the cell.

Interestingly, while ApoB degradation requires BiP, the Hsp70 associated with the ERAD-L pathway, ApoB ERAD also requires the cytosolic Hsp70, Ssa1p, and two Hsp40s, Ydj1p and Hlj1p, that are associated with the ERAD-C pathway (Nishikawa et al., 2005). This may be due to the unusual bitopic nature of ApoB prior to ERAD. My experiments have shown that ApoB interacts with the translocon and is tightly associated with membranes in yeast. One hypothesis for this data is that ApoB can fully translocate into the ER lumen, however in the absence of MTP, the protein lacks lipids and interacts with the nearest hydrophobic surface, the ER membrane. I observed concentrated areas of cytoplasmic staining in addition to the ER localized staining by indirect immunofluorescence, therefore it is likely that when ApoB is expressed in yeast it is found in several different states, partially translocated and associated with translocon, fully translocated and associated with the membrane, and as aggregates in the cytoplasm. In the future it would be important to analyze the topology of ApoB by proteolysis-protection assays on microsomes containing ApoB with differential N and C- terminal tags in order to reveal if any of the N-terminal ApoB is protected by the ER lumen as described in the mammalian system (Pariyarath et al., 2001). Also in order to determine if a portion of ApoB is aggregated in the cytosol of ApoB, solubilization assays can be conducted on pellets from yeast extracts. If ApoB is present in aggregates in the cytoplasm it may be difficult to resolubilize by treatment with mild detergents. However, the aggregates should be dissolved in the presence of stronger detergents, such as SDS. In addition, indirect immunofluorescence and solubilization assays could be conducted with translocation deficient cells, such as yeast containing the temperature sensitive *sec61-2* allele (Pilon et al., 1998), in order to determine if more cytoplasmically localized ApoB and thus ApoB aggregates are observed in cells.

As a component of both the ERAD-L and ERAD-C pathways, the Cdc48p complex is important for the degradation of a variety of ERAD substrates (Bays et al., 2001b; Jarosch et al., 2002; Jentsch and Rumpf, 2007; Nakatsukasa et al., 2008; Rabinovich et al., 2002; Richly et al., 2005; Ye et al., 2001; Ye et al., 2003). Importantly, I found that ApoB degradation was attenuated in yeast containing a mutation in the gene encoding Ufd1p. In addition, I observed reduced ApoB degradation in a strain containing the *cdc48-10* mutant allele (Rabinovich et al., 2002) and in vitro when cytosols were prepared from yeast containing a *ufd1* mutant allele. Ufd1p and Npl4p are adaptors for Cdc48p, which is the yeast homologue of p97/VCP. Cdc48p is a member of the AAA<sup>+</sup> ATPase family that—when coupled with the Npl4p and Ufd1p adaptors—is thought to drive the extraction of poly-ubiquitinated ERAD substrates from the ER membrane and may function as a protein “disaggregase” (Jentsch and Rumpf, 2007). Our lab recently showed that the complex can interact with polyubiquitinated membrane proteins and drive their extraction into the cytoplasm for proteasomal degradation (Nakatsukasa et al., 2008). To date, it is not clear if Cdc48p/p97 directly impacts the degradation of ApoB in mammalian cells, but our data suggest that this complex may function similarly during the disposal of ApoB. Because ApoB is associated with the ER translocon while it is being targeted for degradation it will be interesting to determine if this complex drives the dislocation of ApoB from the translocon for degradation, or if the proteasome is sufficient for this process, as has been observed for other protein substrates (Lee et al., 2004; Wahlman et al., 2007).

## **4.2 HSP110 IS A PROSTABILIZATION FACTOR FOR APOLIPOPROTEIN B IN YEAST AND MAMMALIAN CELLS**

In this dissertation, I identified ApoB as a substrate for Hsp110 and found that the chaperone stabilizes ApoB in vitro and in both yeast and mammalian cells (Figure 40). This result was unexpected given that the yeast Hsp110 homologues, Sse1p and Sse2p, interact with an Hsp70 chaperone that enhances ApoB degradation (Gusarova et al., 2001; Shaner et al., 2005; Yam et al., 2005). Equally surprising was the discovery that other Sse1p-interacting Hsp70s—Ssb1p and Ssb2p—have no effect on ApoB biogenesis. Because all of the Sse1p in the cell may be associated with either Ssa1p or the Ssbs (Shaner et al., 2005), these data suggest that related chaperones or that chaperones within a single complex can act uniquely during the “decision” to protect or degrade a newly synthesized polypeptide at the ER membrane.

How might Sse1p/Hsp110 protect ApoB from proteasome-mediated degradation? Because Sse1p and ApoB co-immunoprecipitate, and because ApoB is targeted for ERAD co-translationally (Benoist and Grand-Perret, 1997; Du et al., 1999; Fisher et al., 1997; Liao et al., 1998; Oyadomari et al., 2006; Zhou et al., 1998), I suggest that the chaperone binds to and shields the substrate as it is being synthesized. This hypothesis is consistent with the reported ribosome-association of Sse1p and the sensitivity of *sse1Δ* mutants to translation poisons (Albanese et al., 2006). This hypothesis is also consistent with the fact that large, hydrophobic loops of untranslocated ApoB are exposed to the cytosol if MTP activity is absent (Mitchell et al., 1998; Pariyarath et al., 2001; Zhou et al., 1998). Such hydrophobic tracts are prime binding sites for chaperones (Rudiger et al., 1997a; Rudiger et al., 1997b; Takenaka et al., 1995), which may be required to retain ApoB in solution and/or target it to the UPP. However, it would be interesting to examine the translation rate of ApoB in yeast and determine if the polypeptide is

translated more slowly than other proteins. This may allow more time for Hsp110 to interact with ApoB. Alternatively, the translation rate of ApoB may be altered in cells lacking Hsp110. Translation rate could be examined by examining the rate of incorporation of S<sup>35</sup> labeled methionine into nascently synthesized ApoB (Purdom-Dickinson et al., 2007). Furthermore, we do not know the translation rate of other mammalian proteins, such as CFTR, in yeast. CFTR translation rate should also be examined as the turnover of this substrate was not altered in *sse1Δ* cells.

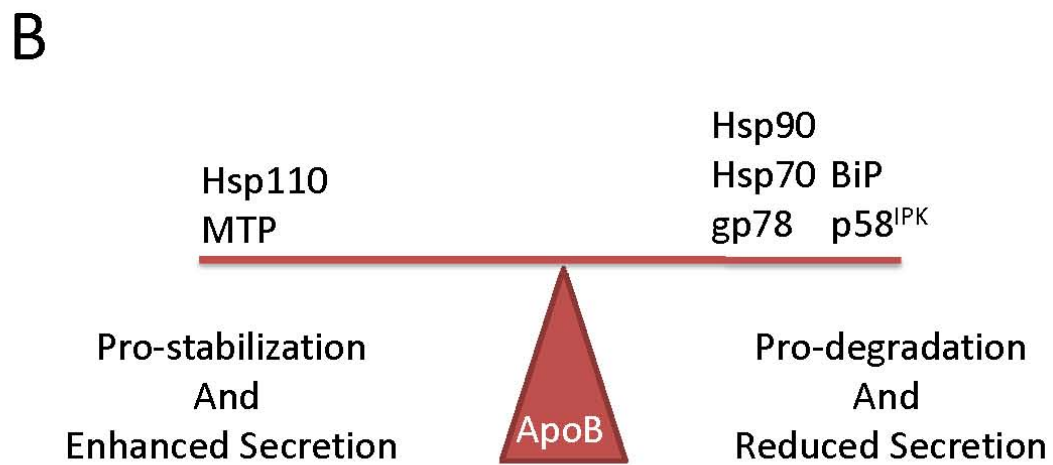
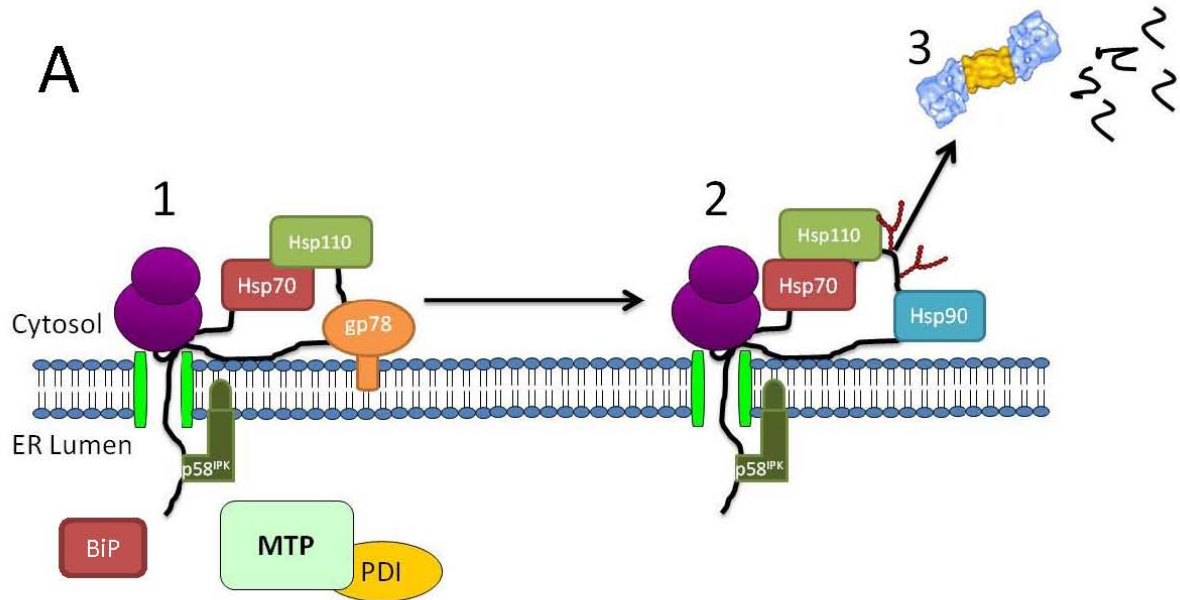
However, Sse1p is not simply protecting ApoB by virtue of its “holdase” activity, which only requires the C-terminal domain in vitro (Goeckeler et al., 2002). Instead, I found that a functional ATP-binding domain is required for Sse1p to stabilize ApoB. Consistent with this observation, it has been previously reported that Sse1p’s ATP-binding domain is required to rescue the temperature-sensitive growth defect of yeast expressing a mutant form of an ER-associated Hsp40 chaperone, Ydj1p (Goeckeler et al., 2002). Therefore, the ATP-binding and hydrolytic activities of Sse1p are critical for substrate protection or to recruit other cytoplasmic factors that may protect newly synthesized ApoB from the UPP. It is tempting to speculate that these events require Sse1p’s recently described nucleotide exchange activity (Dragovic et al., 2006a; Raviol et al., 2006b; Shaner et al., 2005). To begin to address this hypothesis, I examined ApoB ERAD in a strain deleted for two other known Hsp70 exchange factors, *SNL1* and *FES1*, but failed to note any change in ApoB stability.

Because Sse1p directly binds aggregated proteins (Goeckeler et al., 2002; Oh et al., 1997; Oh et al., 1999) and hydrophobic peptide sequences (J. Goeckeler and J.L.B. personal communication), one possibility is that in the absence of Sse1p, more Ssa1p is able to interact with ApoB. In addition, Sse1p may be modulating the chaperone activity of Ssa1p and in the

absence of the Hsp110, the binding of ApoB by Ssa1p may be unregulated. The mammalian Ssa1p homolog, Hsp70, is reported to act upstream of the polyubiquitination machinery in ApoB ERAD and thus is important for both the polyubiquitination and degradation of ApoB (Zhou et al., 1998). If more Ssa1p subsequently associated with ApoB, this may account for the enhanced degradation observed in the *sse1Δ* strain (see Figure 40). Co-immunoprecipitation experiments with Ssa1p and ApoB could be performed in order to determine if the level of Ssa1p associating with ApoB is enhanced in *sse1Δ* cells. In turn, or alternatively, the polyubiquitination status of ApoB may be altered in cells lacking Sse1p. If Sse1p binds ApoB and occludes the polyubiquitination machinery, the absence of the chaperone may result in enhanced ApoB polyubiquitination and increased degradation. The extent of ApoB polyubiquitination could be addressed with both the in vitro and in vivo ubiquitination systems I described in sections 2.1.4 and 3.1.2. In addition, a correlation between Sse1p levels and ApoB polyubiquitination could be further probed by altering the levels of Sse1p both in vivo (with an Sse1p overexpression vector, Figure 27) and in vitro (by supplementing the cytosol with purified Sse1p, Figure 30) because modifying Sse1p levels by both of these methods exhibited a protective effect for ApoB degradation. In addition, experiments in both the in vitro and in vivo system using yeast or cytosol from yeast containing the *ssal-45* temperature sensitive allele and deleted for Sse1p could help determine if enhanced degradation occurs in the absence of both the Hsp70 and the Hsp110. It is unclear however if *ssal-45sse1Δ* yeast are viable.

Based on yeast proteomic and genomic analyses ([www.thebiogrid.org](http://www.thebiogrid.org)), a large number of chaperones and co-chaperones are known to interact with Sse1p. With the exception of Cdc37p, which is involved primarily in kinase maturation (Mandal et al., 2007), I examined each of these





**Figure 40: Molecular Chaperones And ER Luminal Factors Differentially Regulate The Degradation And Secretion Of ApoB In Mammalian Cells.**

A 1: ApoB interacts with ER luminal factors as it translocates into the ER lumen. MTP binds and transfers lipids onto the translocating polypeptide favoring forward translocation. If MTP function is blocked or fatty acid synthesis is limiting, ApoB translocation into the ER stalls, creating a bitopic form of ApoB that is exposed to both the ER lumen and the cytoplasm. The ER luminal factors BiP and p58<sup>IPK</sup> target ApoB for degradation, perhaps by assisting with the retrotranslocation of the polypeptide to the cytoplasm. The cytosolically exposed domains of ApoB are bound by molecular chaperones, Hsp70, Hsp90 and Hsp110. While Hsp70 and Hsp90 are important for ApoB degradation, Hsp110 stabilizes ApoB. The E3, gp78, polyubiquitinates ApoB and the tagged substrate is subsequently degraded by the 26S proteasome. The mechanism by which Hsp110 stabilizes ApoB is unknown, however it may be due to regulating the interaction of Hsp70 with ApoB or restricting access of the other pro-degradation chaperones and the polyubiquitination machinery.

B: ApoB translocation into the ER and assembly into a lipoprotein particle is a finely balanced process. This requires the contribution of factors (MTP and Hsp110) that promote the stability of ApoB to favor the formation of a lipoprotein particle and factors (Hsp70, Hsp90, BiP, gp78, p58<sup>IPK</sup>) that promote degradation of the aggregation prone polypeptide when conditions do not favor lipoprotein particle assembly.

factors (Sti1p, Ssb1p, Ssa1p, Hsp82p, and Ydj1p) for their effects on ApoB degradation. While Hsp82p, Sti1p, Ssa1p, and Ydj1p are reported to function in the Hsp90 complex (Dittmar et al., 1997; Kosano et al., 1998; Smith and Toft, 1993; Smith et al., 1995a; Wegele et al., 2004), Ssa1p and Ydj1p are also reported to function in a disaggregase complex with Hsp26p, Hsp42p, and Hsp104p (Cashikar et al., 2002; Glover and Lindquist, 1998). I did not detect any change in ApoB turnover in cells lacking Sti1p, Hsp26p, Hsp42p, and Hsp104p suggesting that not all components of the Hsp90 and disaggregase complex contribute to ApoB ERAD. However these experiments were conducted with the in vitro system, in which only a small amount of radiolabeled ApoB is associated with microsomes. It may be interesting to revisit the disaggregase complex experiments in the in vivo system, in which much higher levels of protein are produced, to determine if a decrease in the soluble pool of ApoB and altered degradation of the substrate is observed in cells lacking Hsp26p, Hsp42p, or Hsp104p.

Importantly, I observed interactions between ApoB and each of the chaperones that impact ApoB ERAD (Hsp82p, Sse1p, Ydj1p, and Ssa1p). In contrast, I was unable to establish interactions between those chaperones and co-factors that do not contribute to the degradation of ApoB (Sti1p, Sba1p, Ssb1p). A proteomic analysis in mammalian cells indicates that the degradation of wild type CFTR and the disease-causing  $\Delta F508$ -CFTR have distinct co-chaperone interactions and requirements (Wang et al., 2006). These data suggest that each ERAD substrate possesses specific, but likely overlapping chaperone requirements during folding and/or degradation. It should be noted that interactions between Hsp26p, Hsp42p, and Hsp104p and ApoB were unable to be established due to the fact that the antibodies I tested were not immunoprecipitation competent (Hsp26p and Hsp42p) or that ApoB was immunoprecipitated by anti-Hsp104p in the absence of yeast extract suggesting that the antibody cross-reacts with ApoB

or an ApoB-associated protein from the rabbit reticulocyte lysate (data not shown). However, these experiments could be readdressed with the ApoB-chaperone immunoprecipitations using the in vivo system.

The decision to either degrade or stabilize ApoB must be finely balanced because VLDL particles are rapidly assembled and secreted when lipids are abundant; therefore, factors that slow ApoB degradation are vital to regulate the transition between ERAD and VLDL assembly (Tietge et al., 1999). One protein, p58<sup>IPK</sup> (Oyadomari et al., 2006), was recently reported to enhance ApoB degradation but not the turn-over of most other ERAD substrates, and here I report on a factor that instead stabilizes ApoB. Although disabling p58<sup>IPK</sup> function and reducing the activity of the other pro-degradation chaperones, Hsp90 and Hsp70, is predicted to increase VLDL and LDL production—and thus circulating cholesterol levels—Hsp110 inhibition would have the opposite, and desirable, effect (Figure 40). Unfortunately, the LIC peptide identified in our lab as a Sse1p-binding peptide that reduces the ATPase activity of the chaperone did not impact ApoB turnover in vitro (Figure 32). But because fluorescence polarization can be used to assess a fluorescent-LIC peptide and Sse1p interaction (G. Chiosis and J.L.B., data not shown), in principle a high throughput screen for Sse1p inhibitors could be performed in the future.

Importantly, Hsp110 overexpression increased ApoB secretion in rat hepatoma cells in standard media, media enhanced with oleic acid to stimulate ApoB secretion, or media supplemented with an MTP inhibitor to favor ApoB ERAD. This suggests that Hsp110 influences ApoB secretion under several metabolic states and is not limited to a situation when ApoB is rapidly turned over (e.g., minimal fatty acid synthesis or MTP inhibition). In the course of this study, siRNA knockdown experiments to reduce Hsp110 levels in a mammalian cell culture system were unsuccessful due to widespread cell death in the siRNA-treated cells (D.

Habiel, and E.A. Fisher, data not shown). However, due to the degree of similarity between Hsp70 and Hsp110 it is not known if the siRNA constructs utilized were specifically affecting Hsp110 expression or were also modulating Hsp70 levels, thus exacerbating the chaperone deficiency of the cell and inducing cell death. Of note, Hsp70 is strongly anti-apoptotic and a reduction in Hsp70 levels is lethal (Li et al., 2000; Mosser et al., 2000; Nylandsted et al., 2000). However, future research efforts to address the physiological impact of Hsp110 on ApoB secretion in a mammalian system could be addressed in mice. Mice are reported to express three Hsp110 isoform (Hsp105, Apg-1, Apg-2) (Kaneko et al., 1997a; Kaneko et al., 1997b; Yasuda et al., 1995). If mice containing deletions for each of these genes individually or in combination were obtained they could be examined for lower circulating levels of ApoB in their serum compared to the control strain. In addition, the animals could be stressed with a high fat diet to determine if they are more resistant to plaque formation and developing CAD.

## **APPENDIX A**

### **IDENTIFYING COMPONENTS OF APOLIPOPROTEIN B-PROTEIN COMPLEXES IN YEAST BY MASS SPECTROMETRY**

A previous study identified ApoB interacting proteins from cross-linked mammalian cell extracts by mass spectrometry (Rashid et al., 2002). The proteins identified included a multitude of ribosomal proteins and a few ER luminal factors (PDI, glutathione-S-transferase, and BiP). However, Hsp70 and Hsp90, the two cytosolic chaperones involved in ApoB ERAD, were not identified in the cross-linked precipitate (Fisher et al., 1997; Gusarova et al., 2001; Zhou et al., 1995). Because large amounts of protein extracts can be obtained from yeast expression systems and because I have shown that an isoform of the human ApoB protein can be expressed and immunoprecipitated from yeast extract, I chose to use mass spectrometry to identify ApoB-associated proteins in yeast. In this section I will describe the immunoprecipitation conditions I tested to optimize ApoB-chaperone complex isolation for mass spectrometry. In addition, I will list a few ApoB interactors I identified by mass spectrometry, including the cytosolic Hsp70, Ssa1p. Finally, I will describe a new *GAL4* overexpression strain that greatly increases the level of ApoB expression induced in yeast, a limiting factor for the immunoprecipitation experiments.

## **A.1 METHODS AND MATERIALS**

### **A.1.1 Immunoprecipitation Technique**

For ApoB immunoprecipitation experiments with the anti-HA resin (Roche), cell extracts from 200 ODs of yeast were prepared by 5, 1min agitations with glass beads in Roche lysis buffer (50mM Tris, pH 7.5, 25mM NaCl, 0.1% Nonidet P40, 20mM NaMoO<sub>4</sub>) with protease inhibitors (0.25mM MG132, 1mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin A) with the reactions cooling on ice in between agitations. The lysate was centrifuged for 5min at 5000rpm at 4°C. The supernatant was removed to a fresh 1.5mL eppendorf tube and the protein concentration was determined by analyzing 5µl of extract in 1mL of 2% SDS by A<sub>280</sub>. Next, 10mg of cell extract was diluted five-fold in Roche lysis buffer with protease inhibitors (0.25mM MG132, 1mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin A) and supplemented with 20uL of an ATP regeneration mixture (1mM ATP, 40nM creatine phosphate, and 0.2mg/mL creatine phosphokinase). The lysate was then incubated for 4h at 4°C with 150µL of the anti-HA resin or sepharose 6B beads in the Roche lysis buffer that were used as a negative control (Sigma). The beads were washed 3 times with 700µl of cold Roche lysis buffer containing 150mM NaCl with the beads pelleted at 3000rpm at 4°C for 2min. The reactions were then washed an additional 3 times with 700µl of cold Roche lysis containing 300mM NaCl. All of the supernatant was removed with a gel loading tip. Finally, the beads were eluted with 120µL Urea buffer (125mM Tris, pH 6.8, 4% SDS, 6M urea, 1mM EDTA, 10mM DTT, 250mM β-mercaptoethanol, 20% glycerol, 0.05% bromophenol blue). ~10µL of the precipitate was resolved by SDS-PAGE and

the relevant proteins were identified by immunoblot analysis as described above (Chapter 2.1). The remaining eluate was utilized for mass spectrometry (see below).

### **A.1.2 Gel Staining And Mass Spectrometry**

Approximately 90 $\mu$ l of the isolated protein precipitates were resolved by SDS-PAGE using a 1.5mm thick 7 inch x 8 inch 10% polyacrylamide gel. The gel was then stained with a colloidal blue kit (Invitrogen, 60mL double distilled water (ddH<sub>2</sub>O), 30mL Methanol, 20mL Stainer B, 40mL Stainer A) for 48h. The gel was then destained in sterile ddH<sub>2</sub>O for ~4h. The bands containing the resolved proteins were excised with a methanol washed razor blade. One-third of the band was placed in the 96 well plate for trypsin digestion and the remaining two-thirds were set aside in an 1.5mL eppendorf tube as a back-up for further analysis. The samples were taken to the Minden laboratory at Carnegie Mellon University and with the assistance of Dr. Susan Dowd the plate containing the gel slices was loaded into the ProGest automated peptide digestion system for trypsin digestion. Following trypsin digestion, the peptide samples were bound to the resin tip of the ZipTip C18 system (Millipore), washed with 0.2% formic acid, and eluted with CH<sub>3</sub> CN- 0.2% formic acid (1:1). The peptides were then analyzed by matrix assisted laser desorption/ionization- time of flight (MALDI-TOF) mass spectrometry as previously described (Gong et al., 2004). Data analysis was conducted using MASCOT software (Perkins et al., 1999).



### A.1.3 ApoB *GAL4* Overexpression System

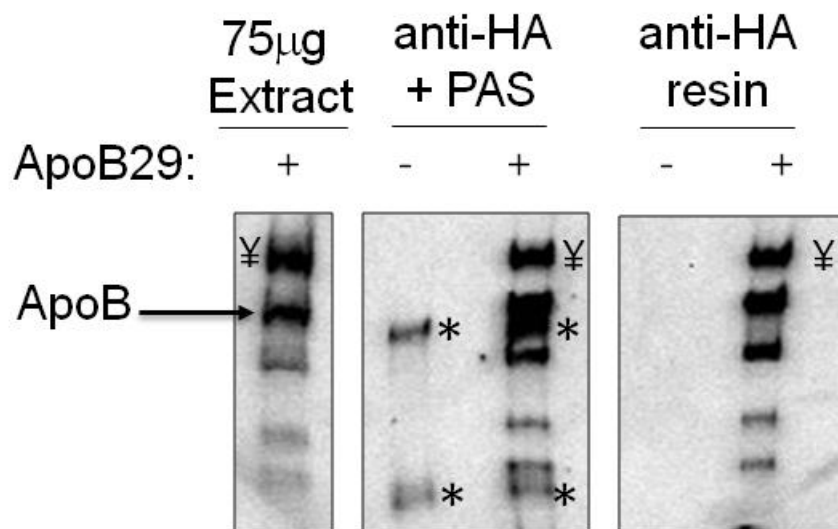
A W303.1b strain was obtained that was modified to carry one additional chromosomal copy of the *GAL4* transcription factor driven by a *GAL10* promoter (Lenoir et al., 2002). The additional chromosomal copy is *TRP1* marked and the resultant strain is: *MAT a*, *leu2*, *his3*, *ura3*, *ade2-1*, *can<sup>r</sup>*, *cir<sup>+</sup>*, *trp1::TRP1-GAL10-GAL4*. The strain RSY620 (*MAT a*, *ade2-1*, *trp1-1*, *leu2-3,112*, *ura3-1*, *his3-11,15*, *pep4::TRP1*) was utilized as a control strain with a single chromosomal copy of the *GAL4* gene. ApoB expression was induced as described in Chapter 2.1. Approximately 10 ODs of cells were taken at the following timepoints in the induction: 0, 2, and 4h. Microsomes were prepared by 5, 1 min agitations with glass beads in 200µl lysis buffer (20 mM HEPES, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.1 M sorbitol, 1 mM DTT) with protease inhibitors (0.25mM MG132, 1mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin A). The supernatant was moved to a fresh 1.5mL eppendorf tube and the glass beads were washed twice with 500µl of Buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) with protease inhibitors (0.25mM MG132, 1mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin A). The washes were pooled with the cell extracts and cell debris was removed by centrifuging the extracts at 3000rpm for 3min at 4°C. The supernatant was moved to a fresh tube and the extract was centrifuged again using the same conditions to insure all cell debris was removed. The resulting supernatant was moved to a fresh 1.5mL eppendorf tube and centrifuged at 14 000rpm for 10min at 4°C to pellet the microsomes. The microsomes were then resuspended in 50µl of Buffer88 and the protein concentration was determined by analyzing 5µl of extract in 1mL of 2% SDS by A<sub>280</sub>. Approximately 75µg of microsomes were resuspended in 2x urea buffer, (see A.1.1), heated for 3min at 70°C and resolved by SDS-PAGE and

immunoblotted with the indicated anti-sera as previously described. Unlike the expression in a traditional wildtype strain, it should be noted that ApoB expression was able to be easily detected in the *GAL4* overexpression strain using the Supersignal West Pico Chemiluminescent Substrate (Pierce).

## **A.2 RESULTS**

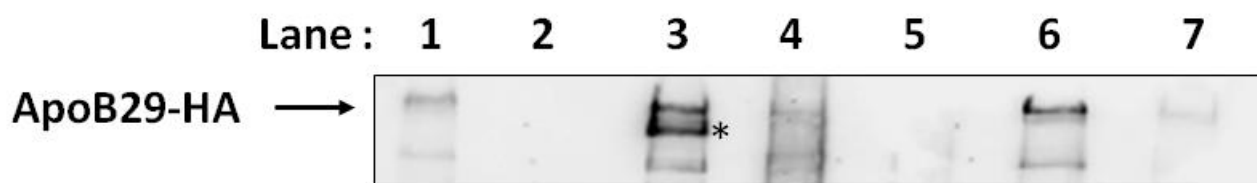
### **A.2.1 ApoB29-HA Can Be Immunoprecipitated With Both Anti-HA And Anti-ApoB Antibodies**

In order to examine ApoB-chaperone complexes I needed to optimized the immunoprecipitation conditions. First, I examined the efficiency of ApoB precipitation with the HA-conjugated resin versus the unconjugated, free HA antibody with protein A sepharose. Importantly, the efficiency of both antibody treatments was similar, immunoprecipitating approximately 38% of the total ApoB from the extract (Figure 41). In addition, the conjugated resin did not appear to significantly release any antibody from the beads, thus reducing the immunoglobulin background bands on the western blot (indicated by \*). In addition, I examined the competence of a variety of rat and human antibodies for ApoB immunoprecipitation from yeast extracts (Figure 42). The goat anti-human ApoB antibody exhibited the best immunoprecipitation competence, however the HA antibody was still the best candidate for significant immunoprecipitation of HA tagged ApoB29 from yeast extract. Finally, I wanted to examine the precipitates when using two previously described wash conditions (Section 2.1.3) and the anti-HA resin to determine if any differences could be observed between the precipitated protein pools. When visualized by silver



**Figure 41: ApoB Immunoprecipitation Efficiency With Anti-HA Conjugated Resin And Unconjugated Anti-HA With Protein A Sepharose**

Extracts were prepared from cells transformed with a vector control (-) or with pSLW1-B29 (+) and were treated with anti-HA resin or unconjugated anti-HA with protein A sepharose (PAS). The beads were washed with buffers containing 150mM and 300mM NaCl. The proteins in the precipitates were resolved by SDS-PAGE and were immunoblotted with the indicated antisera. Anti-HA cross reactive bands in the unconjugated anti-HA immunoprecipitation observed in ApoB containing extracts and the vector control are denoted with an \*. The HA cross reactive band that is larger than ApoB29 and has been previously detected is denoted with a ¥.



- 1 30μg cell extract
- 2 No Antibody
- 3 Anti-HA
- 4 Anti- human ApoB (1D1 mouse)
- 5 Anti- rat ApoB (goat)
- 6 Anti- human ApoB (goat)
- 7 Anti- rat ApoB (rabbit)

**Figure 42: ApoB Antibodies Can Immunoprecipitate ApoB29 From Yeast Extracts**

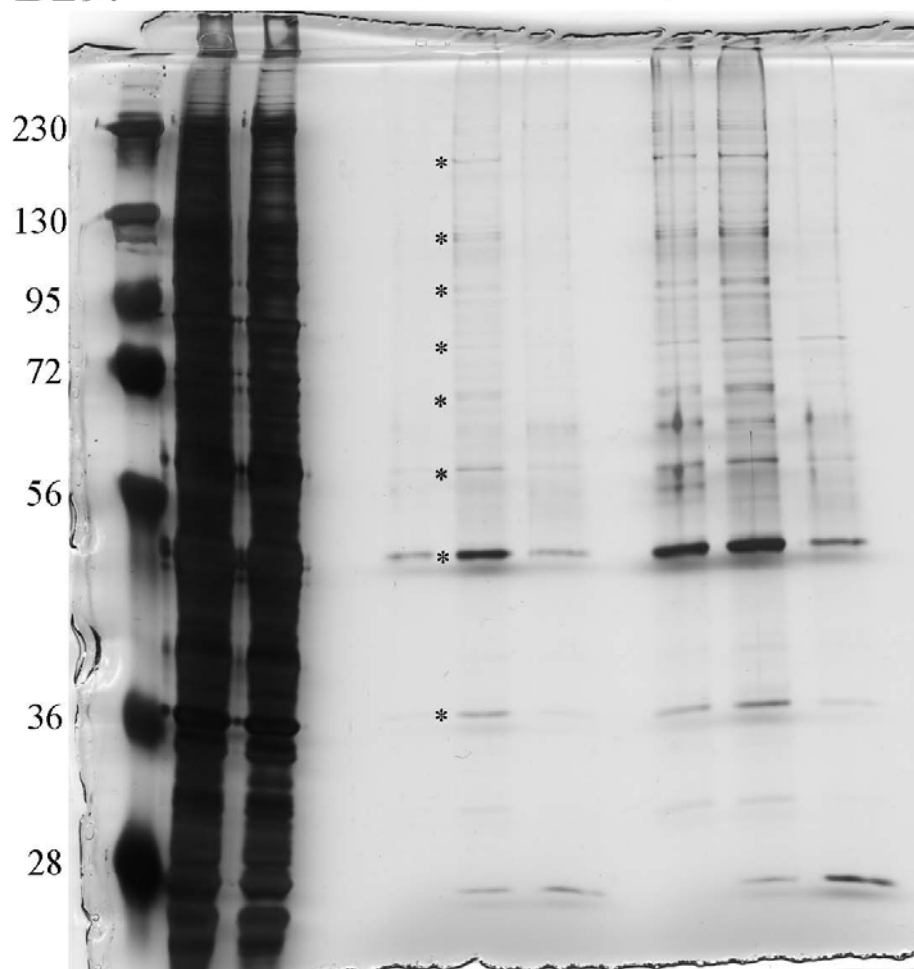
A) Extracts were prepared from cells transformed with a vector control or with pSLW1-B29 (B29) and were treated with unconjugated anti-HA with protein A sepharose, a protein A sepharose control, anti-human ApoB antibody (1D1, mouse, a kind gift from Ross Milne, The University of Ottawa) with protein A sepharose, anti-rat ApoB (goat, a kind gift from Edward A. Fisher, New York University School of Medicine) with protein A sepharose, anti-human ApoB (goat, Calbiochem) with protein A sepharose, and anti-rat ApoB (rabbit, a kind gift from Dr. Janet Sparks, University of Rochester School of Medicine) . The beads were washed with buffers containing 150mM and 300mM NaCl. The proteins in the precipitates were resolved by SDS-PAGE and were immunoblotted with anti-HA. Note the cross reactive band that appears when anti-HA is included in the precipitation reaction is denoted with an \*.

stain, the salt washed immunoprecipitates exhibited differences in both the amount of protein and protein composition when comparing the ApoB29HA immunoprecipitation reactions and the vector and unconjugated resin controls (Figure 43). The Tween20-washed immunoprecipitations had significantly more overlap in the protein pools and higher background when using the unconjugated resin control. Therefore, for subsequent mass spectrometry experiments, I utilized the salt wash conditions for the immunoprecipitations.

### **A.2.2 Mass Spectrometry Data For ApoB29 Immunoprecipitated From Yeast Extracts**

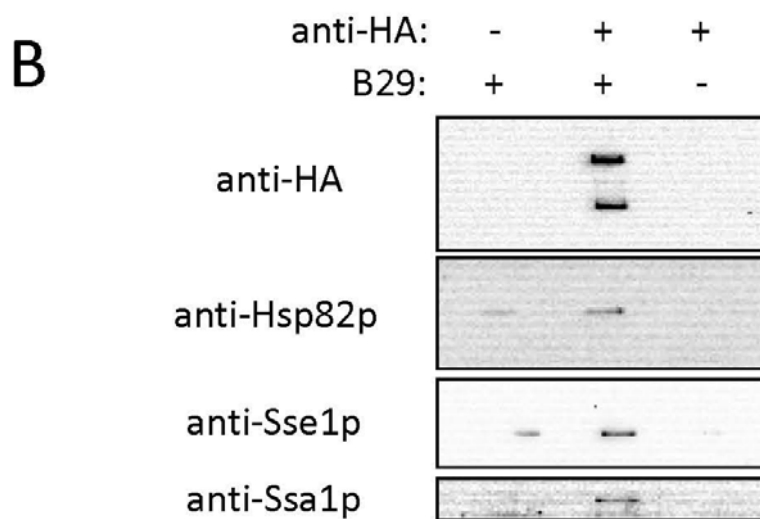
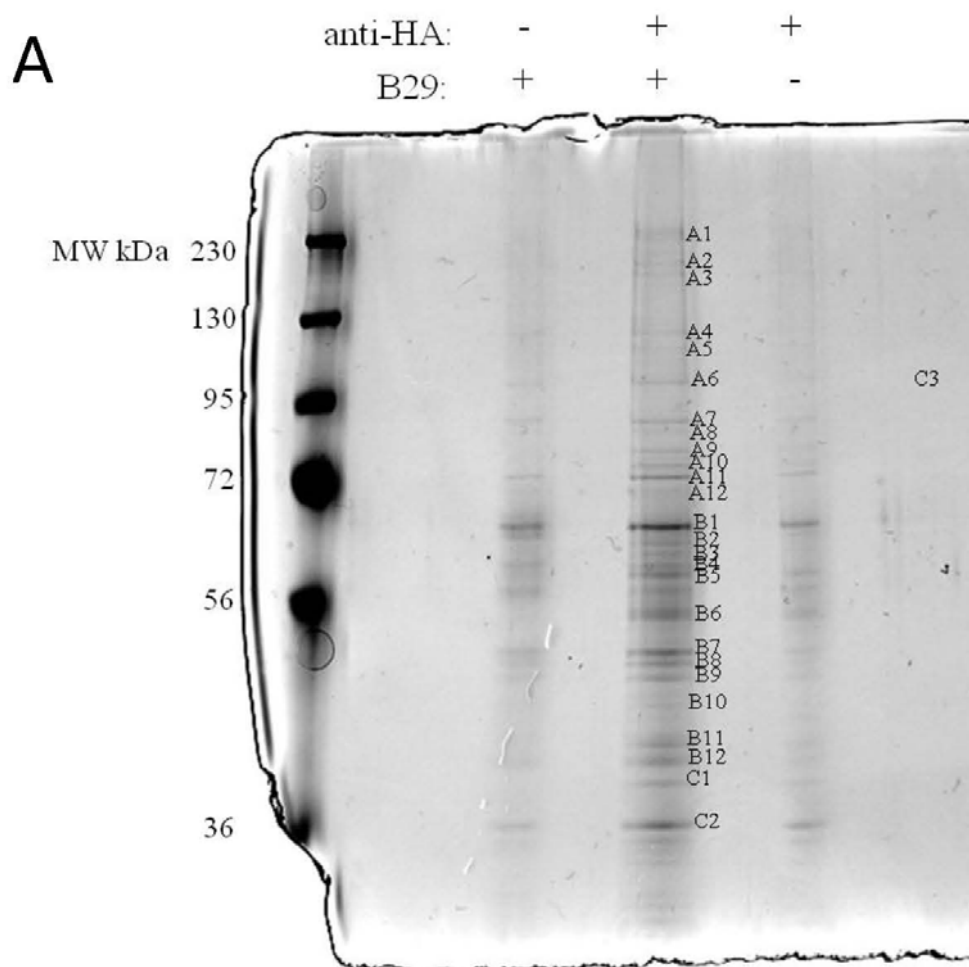
ApoB was immunoprecipitated from cell extracts and stained with a colloidal blue kit (Invitrogen) as described above (Section A.1.2). The stained gel with the resolved precipitated proteins are depicted in Figure 44A. Importantly, the Hsp82p, Sse1p, and Ssa1p cytosolic chaperones were detected in the precipitate by western blot analysis (Figure 44B). However, it should be noted that some chaperones were also observed in the precipitate with the bead only control. This higher background may be due to insufficient washing in the scaled-up immunoprecipitations (from 1mg to 13.5mg of total protein in the precipitation). The indicated protein bands were excised and analyzed using MALDI-TOF mass spectrometry. The C3 gel area was utilized as a control to determine contaminating peaks from the agarose gel alone. The proteins identified with a probability-based MOWSE score of greater than 61 are considered significant (Perkins et al., 1999) and are listed in Table 8. While the cytosolic Hsp70, Ssa1p, was identified in the precipitate, the majority of proteins identified are metabolic enzymes. However, the isolation of a protein of unknown function (Ydr051cp) that is reported to interact with Hsp82p (<http://db.yeastgenome.org/cgi-bin/locus.pl?locus=ydr051c>) is an intriguing result.

	Extract			Salt Wash			Tween 20 Wash		
	(10% of Protein in IP)			(150mM + 300mM)			(0.5% + 1%)		
anti-HA:	-	-	-	-	+	+	-	+	+
B29:	-	+	+	+	+	-	+	+	-



**Figure 43: Silver Stain Of ApoB Immunoprecipitations With Different Wash Conditions**

Extracts were prepared from cells transformed with a vector control (-) or with pSLW1-B29 (+) and were treated with anti-HA resin (+) or an unconjugated sepharose resin control (-). The resin was washed with buffers containing 150mM and 300mM NaCl or with buffers containing 0.5% Tween20 and 1% Tween20. The proteins in the precipitates were resolved by SDS-PAGE and visualized by silver stain. Proteins enriched in the salt washed sample compared to the control reactions are marked with an \*.





**Figure 44: ApoB Gel and Westerns For Mass Spectrometry Samples**

A) Extracts were prepared from cells transformed with a vector control (-) or with pSLW1-B29 (+) and were treated with anti-HA resin (+) or an unconjugated sepharose resin control (-). The resin was washed with buffers containing 150mM and 300mM NaCl. The proteins in the precipitates were resolved by SDS-PAGE and visualized by colloidal blue stain. Note that there is protein bound in the control precipitates, however the bands are enriched in the ApoB containing anti-HA resin precipitates.

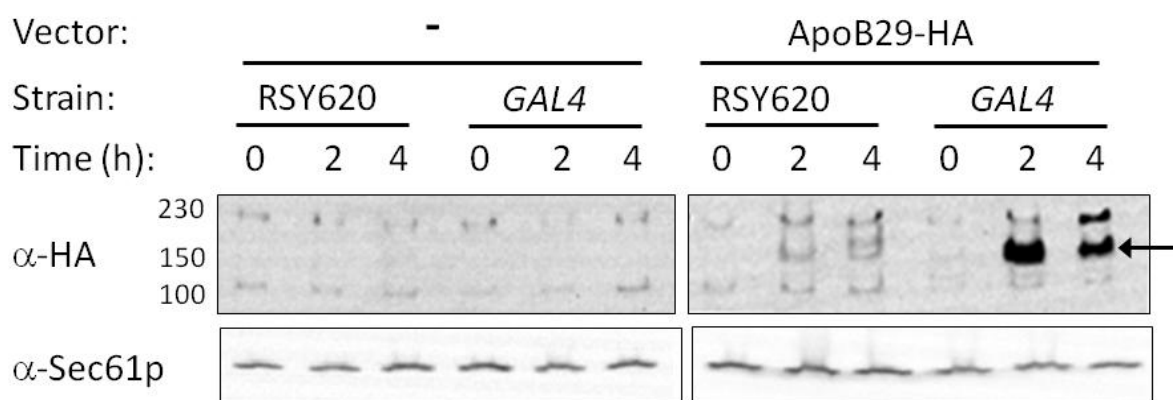
B) The proteins in the precipitates from A were resolved by SDS-PAGE and were immunoblotted with the indicated antisera. Note that control precipitates contain a small amount of Sse1p and Hsp82p.

**Table 8: ApoB Interacting Proteins Identified By Mass Spectrometry**

Band	Size, kDa	Gene	Function	% Coverage	Score	# Peaks
A11	70	Ssa1p	Cytosolic Hsp70	17	81	9
B2	54	Ald1p	Cytsolic aldehyde dehydrogenase	20	73	7
B3	61	Yel020cp	Hypothetical protein with low sequence identity to Pdc1p, a protein involve in amino acid catabolism	26	73	11
B8	47	Eno1p	Enolase II, amino acid transport and metabolism	21	74	6
B9	45	Pgk1p	Phosphoglycerate kinase	23	66	8
C1	42	Bat2p	Amino Acid Aminotransferase	26	84	7
C2	39	Ydr051cp	Protein of unknown function, interacts with Hsp82p in a two- hybrid assay	26	70	7

### **A.2.3 The GAL4 Overexpression System Enhances The Induction of ApoB Expression In Yeast**

One significant issue with the mass spectrometry results is the failure to identify a band containing ApoB by mass spectrometry or observe a significantly enriched band corresponding to the molecular weight of ApoB29-HA on the gel. However, I can detect ApoB in the precipitate by western blot analysis (Figure 44B). In order to increase the available pool of ApoB for precipitation, and thus hopefully enhance the probability of detecting ApoB in the precipitate, I examined ApoB expression in a yeast strain engineered to have higher levels of galactose inducible protein expression (Lenoir et al., 2002). The W303.1b strain contains an additional copy of the *GAL4* transcription factor under the control of the *GAL10* promoter integrated into the chromosome. When I examined ApoB expression in microsomes obtained from the W303.1b-*GAL4* strain and another yeast strain (RSY620) that I have used to study ApoB degradation (Figure 19B) I observed 12 fold more ApoB expression in the *GAL4* overexpression strain (Figure 45). In addition, the induction time was much faster in the *GAL4* overexpression strain, peaking at 2h compared to the 4-6h peak induction times I had previously observed in wildtype W303.1b (data not shown). The ApoB signal in the microsomes in Figure 45 was visualized by developing the immunoblots with the Supersignal West Pico Chemiluminescent Substrate (Pierce) and did not require the more sensitive Supersignal West Femto Chemiluminescent Substrate (Pierce) for detection.



**Figure 45: ApoB Expression In A *GAL4* Overexpression Strain**

Microsomes were prepared from cells transformed with a vector control (-) or with pSLW1-B29 (ApoB29-HA) after induction in YP-GAL at the indicated timepoints. Two different strains were utilized for ApoB expression: RSY620, a standard wildtype strain, and a W303.1b strain with one extra chromosomal copy of the *GAL4* transcription factor. Approximately 75 $\mu$ g of protein from the microsomes was resolved by SDS-PAGE and immunoblotted with the indicated antisera. Sec61p was visualized as a loading control for the microsomes. The expression of ApoB (denoted with an arrow) is maximally induced at 2h in the *GAL4* overexpression strain.

### **A.3 FUTURE DIRECTIONS**

In the preceding section I described attempts to optimize immunoprecipitation and ApoB expression conditions in order to obtain mass spectrometry data for ApoB-chaperone complexes. In the future, I will utilize two approaches to reduce the increased background observed in the control reactions of the scaled up immunoprecipitation experiments. First, I will conduct several small scale immunoprecipitations with 1mg of protein in unison and then pool the precipitated proteins in order to obtain enough protein to analyze by mass spectrometry. Second, as many of the proteins identified were cytosolic, metabolic proteins, I will conduct immunoprecipitations using both cell extracts and solubilized microsomes in order to determine if immunoprecipitating ApoB from microsomes increases the proteins that may specifically associate with ApoB. Finally, these immunoprecipitations will be conducted in the *GAL4* overexpression strain, as increasing the available pool of ApoB for immunoprecipitation may be critical to successfully identify associated polypeptides.

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